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TITLE: Modulators of Response to Tumor Necrosis-related Apoptosis Inducing Ligand (TRAIL) Therapy in Ovarian Cancer

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14. ABSTRACT Ovarian cancer is the leading cause of death from gynecologic cancers in the developed world. We have previously identified a homeobox gene, Six1, which is overexpressed in ovarian cancers as compared to normal ovarian surface epithelium. We have shown that overexpression of Six1 is associated with resistance to Tumor Necrosis Factor-related Apoptosis Inducing Ligand (TRAIL) based therapies, both in cell culture and in patient tumors. We have also discovered that elevated Six1 is associated with tumor formation in mice and that the TRAIL decoy receptor DcR2 is overexpressed when Six1 is overexpressed. However, knockdown of DcR2 does not restore TRAIL sensitivity to Six1 overexpressing tumors, implying additional mechanisms. On-going studies are evaluating the mechanism and significance of these findings on the way to designing new treatments for ovarian cancer.					
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INTRODUCTION:

Ovarian cancer is the leading cause of death from gynecologic cancers in the developed world. Most ovarian cancers are diagnosed late and current treatment results only in a 20% 5-year survival in advanced disease. More effective therapies are urgently needed. One of the most promising therapies in development for ovarian cancer is the use of either the Tumor Necrosis Factor-related Apoptosis Inducing Ligand (TRAIL) or agonistic antibodies that activate the receptors for TRAIL. Both these strategies are designed to induce apoptosis in ovarian cancer cells. TRAIL therapies are particularly exciting because TRAIL reverses chemoresistance to standard chemotherapy as well as having a direct growth inhibitory effect on ovarian cancer cells, while sparing normal ovarian cells. However, the characteristics of ovarian tumor cells that determine whether TRAIL pathway agonists will be effective are poorly understood. For this reason, we currently do not have a rational basis for selecting patients who will benefit most from drugs that target this pathway or for improving the clinical response in those patients whose tumors are refractory to TRAIL pathway activators.

We have previously identified a homeobox gene, Six1, which is over-expressed in ovarian cancers as compared to normal ovarian surface epithelium. Expression of Six1 is correlated with poor clinical prognosis and confers resistance to TRAIL, possibly via upregulation of a decoy receptor. Our original hypothesis was that **“Six1 expression in ovarian cell lines and primary tumor cells results in resistance to TRAIL-induced apoptosis through activation of the DcR1 decoy receptor”**. In the first year of the award, DcR1 expression in relation to various Six1 over-expression systems was evaluated and was not found to correlate with Six1 over-expression. However, a related TRAIL decoy receptor, DcR2 was found to increase in Six1 over-expressing cells. In the third year of the award we attempted to determine if DcR2 was a direct or indirect target of Six1. To analyze the likelihood of DcR2 promoter binding by Six1, mRNA upstream of the DcR2 translation site was examined for the presence of the “TCAGG/CCTGA” consensus Six1 binding sequences. Four such regions were identified and an electrophoretic mobility assay confirmed that these sequences in the DcR2 promoter interact with Six1. However, knocking down DcR2 did not sensitize cells to TRAIL. It was also found that increased DcR2 in Six1 expressing cells correlates with increased DR5 such that the cell compensates for the increased decoy receptor DcR2 by increasing the functional death receptor DR5. This suggested that increases in DcR2 related to increased Six1 are not the mechanism for TRAIL resistance. This completed tasks 1 and 2 (additional detail is presented in upcoming sections).

To complete Task 3, we attempted to develop inducible Six1 overexpression and knockdown systems. None of the approaches resulted in reproducible or tightly controlled effects. Six1 knockdown in particular frequently resulted in cancer cell lines that did not grow. In the absence of an inducible system, stable CaOv3-Six1 overexpressing clones were generated and the phenotype of TRAIL resistance was reconfirmed.

To complete Task 4, in year two of the award we reported that we had enrolled 29 patients and had established 17 primary ovarian cancer cell lines from those patients. We tested the cell lines for tumorigenicity in CB-17 SCID mice, Six1 expression and TRAIL sensitivity. 6/17 cell lines

generated tumors and tumorigenicity was correlated with increased Six1 expression (χ^2 p=0.02). Although there was no correlation between Six1 level and TRAIL sensitivity in the primary tumors, we discovered that primary cell lines as well as the majority of mice tumors lose Six1 expression with *in-vitro* and *in-vivo* passage, hence the Six1 level of the tumors was not reflective of the Six1 level of the cell line at the time it is being tested for TRAIL sensitivity. As an alternate approach, we developed an *ex-vivo* tissue slice system for analyzing TRAIL sensitivity in patient tumors (as opposed to establishing cell lines from those tumors) in year three and report the results in this final report.

Task 5 was discontinued after it was found that only one of ten mouse ovarian cancer cell lines overexpressed Six1. Hence Six1 was likely not an important contributor to mouse ovarian cancers and a syngeneic mouse model for Six1 related ovarian carcinogenesis was unlikely to contribute to the understanding of human Six1 related ovarian carcinogenesis.

Our preliminary experiment for Task 6 was to study growth rates of CaOV3 CAT and CaOV3-Six1 transfectants on the flanks of 4-6 week old CB-17 SCID mice. 4 clones total, 4 mice/group and two tumors/ mouse were initiated by injecting 1×10^7 cells and observing for tumor growth with biweekly measurements of tumor size. Six1 expressing tumors measured $38 \pm 6 \text{ mm}^3$ at 2 weeks as compared to $7 \pm 3 \text{ mm}^3$ for CAT clones (p<0.001 t-test) demonstrating a faster initial growth rate for Six1 expressing tumors. However tumor growth was poor in the subsequent weeks for both CAT clones and Six1 clones. At 10 weeks Six1 expressing tumors measured $50 \pm 19 \text{ mm}^3$ as opposed to $29 \pm 11 \text{ mm}^3$ for CAT expressing clones. This difference was no longer significant. A subsequent experiment demonstrated that the CaOV3-Six transfectant xenograft tumors lost Six1 expression within 2 weeks, associated with a decrease in growth rate to baseline. Attempts to study the SKOV3 Six1 knockdown clones in the same system resulted in robust tumor growth in the SKOV3 parental line, but no tumor growth in the SKOV3 Six1 siRNA tumors. These findings were encouraging because they highlighted the importance of Six1 in maintaining tumor growth. However, loss of Six1 over-expression and the lack of tumor growth in knockdown clones made the study of the effects of treatment in this system difficult. Hence we favored development of the *ex-vivo* model as suggested in task 4 to complete this task as well. Data is presented under task 4.

Because our experiments were designed to generate data that would be helpful in the design of phase I studies of TRAIL and its agonistic antibodies in cancers (ovarian and others), we have performed additional experiments aimed at the mechanism of TRAIL resistance in Six1 over-expressing cells. These are the subject of current (see reportable outcomes) and planned manuscripts. We are underway to predict which cancers are TRAIL insensitive by virtue of their levels of Six1 expression, providing a way to select patients for TRAIL clinical trials that are more likely to benefit from this therapy. Furthermore, many currently used chemotherapeutic agents exert their cytotoxic effect through activating the TRAIL pathway and TRAIL therapy is synergistic with many chemotherapies. Hence, TRAIL resistance may be a marker for chemotherapy resistance and over-coming TRAIL resistance may render cells sensitive to chemotherapy. Since development of chemoresistance is a major obstacle to successful ovarian cancer therapy, a natural extension of our findings in a subsequent proposal would be to study the effects of reversing TRAIL resistance on the effectiveness of chemotherapy.

BODY:

This section is organized according to the proposed statement of work for the award and outlines detailed accomplishments towards completing the task.

Task 1. Verify DcR1 (DcR2) as a target of Six1 (1-9 months) – Completed.

Stable-expressing CaOV3-CAT clones CaOV3-Six1 clones were generated early in this task for a more-robust analysis. Analysis of CaOV3-Six1 over-expressing cell lines and cell lines from ovarian cancer patients did not confirm DcR1 as a downstream target (see Figures 1-4 below). Furthermore, the SKOV3 cell line, which over-expresses Six1, did not over-express DcR1, making siRNA experiments not possible. However, another decoy receptor, DcR2 was up-regulated and studied further. Data leading to this conclusion is presented.

Figure 1. 3 CaOV3 CAT clones are compared to 4 CaOV3-Six1 over-expressing clones. Six1 and DcR1 expression is evaluated by quantitative real-time PCR (reported as fgSix1/ng 18s rRNA, Y Axis). Six1 transfected CaOV3 clones (last 4 data sets) have greater Six1 expression (first bar, red) but not greater DcR1 expression (second bar, blue) as compared to CAT controls (first 3 data sets).

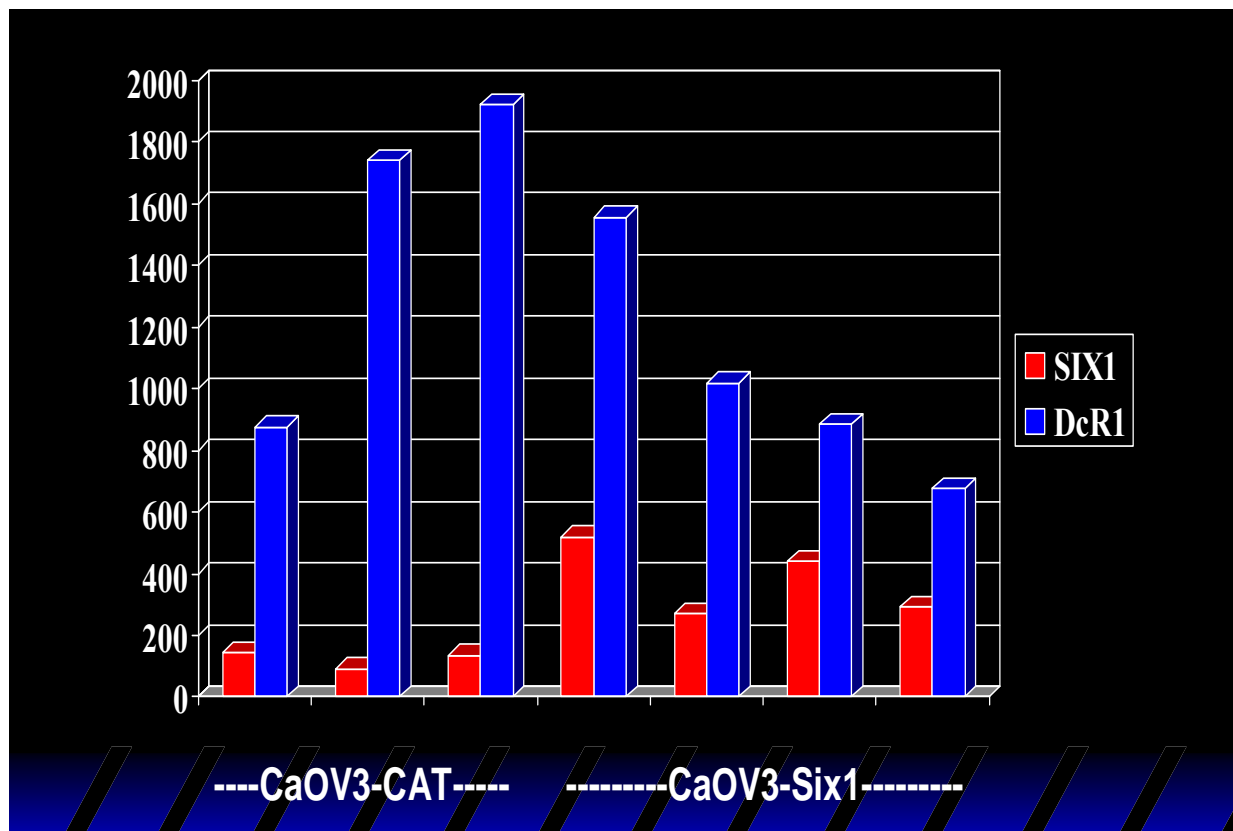


Figure 2. 2 newly generated CaOV3-CAT clones are compared to 2 newly generated CaOV3-Six1 clones and the Six1 over-expressing cell Line SKOV3. TRAIL receptor analysis is performed by flow cytometry (Y-Axis = % of cells positive). While DcR1 over-expression is not consistent in CaOV3-Six1 over-expressing cells, DcR2 is overexpressed, making it a better possible downstream target. The SKOV3 cell line also over-expresses DcR2.

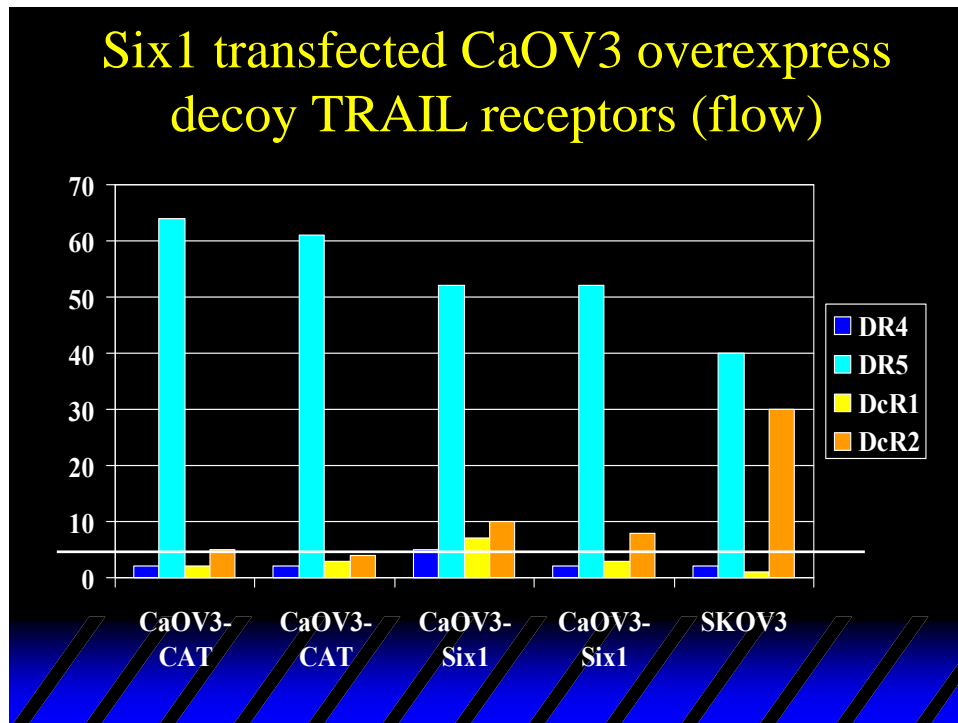


Figure 3. In support of Figure 2, analysis of DcR2 protein by western blot also demonstrates that DcR2 is over-expressed in CaOV3-Six1 clones. A HeLa cell line known to express DcR2 is included as control.

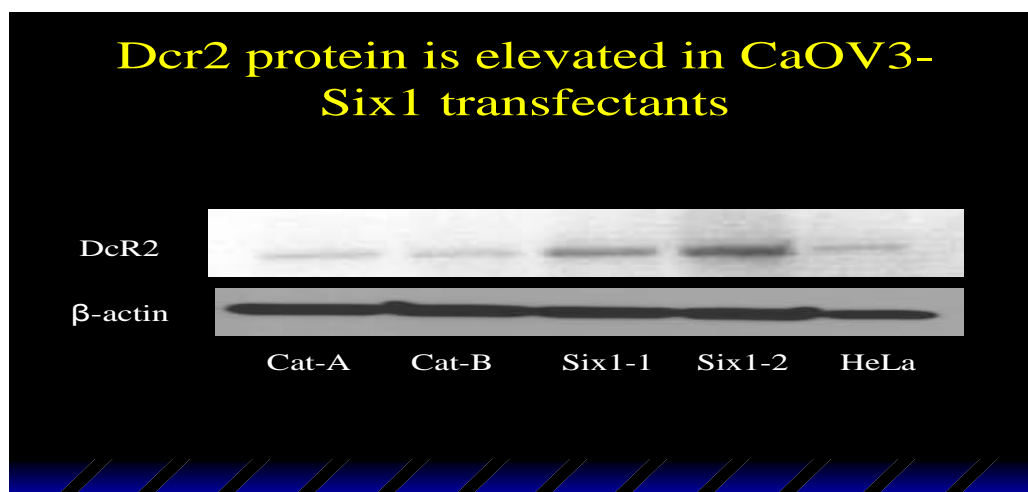
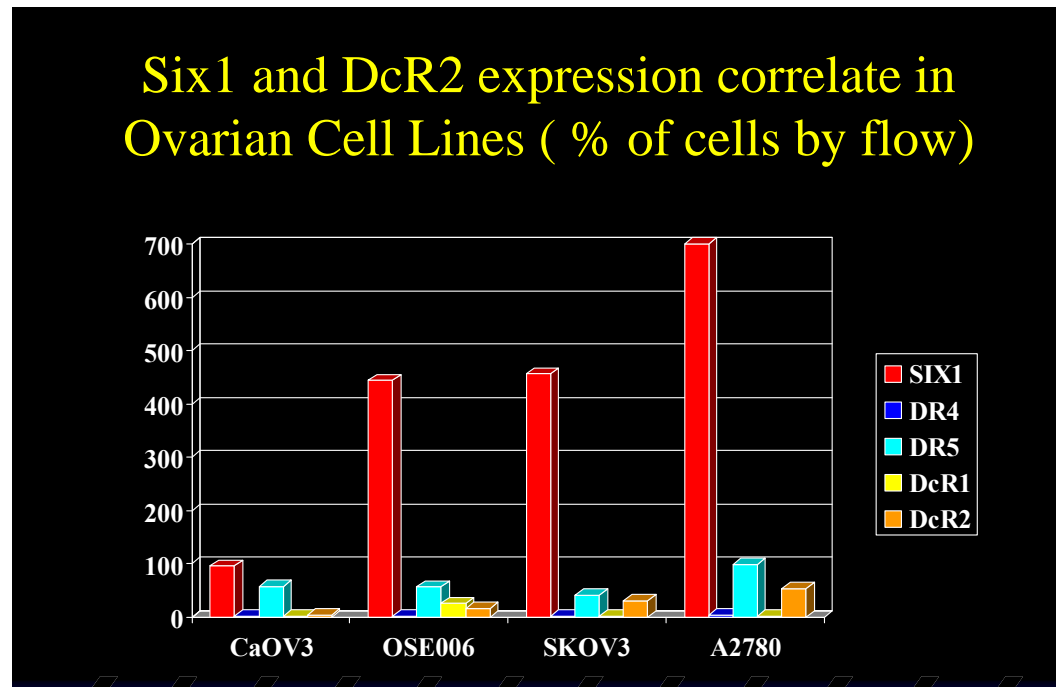


Figure 4. Analysis of additional commercially available cell lines shows a correlation between Six1 over-expression (first, tallest bar, red, Y Axis = fg Six1/ng 18s rRNA) and DcR2 overexpression (last bar, orange, Y axis = % cells positive by flow cytometry). Percent cells positive by flow cytometry for the TRAIL receptors DR4, DR5 and the Decoy receptor DcR1 are also shown (bars 2, 3 and 4 respectively).



Due to the lack of correlation between DcR1 and Six1 and initial data showing a positive correlation between DcR2 and Six1, this task was modified going forward to study the correlation between Six1 and DcR2. Hence the specific tasks became:

- Collect and propagate specimens and cell lines to complete Six1 RNA and *DcR2* RNA and protein analysis.
- Perform CaOV3-Six1 and SKOV3 SiRNA experiments.

This task was completed and reported on the year 2 report as follows:

A panel of ovarian cancer cell lines with various endogenous Six1 levels [1-7] were propagated and analyzed for Six1 expression and DcR2 expression by quantitative real time PCR (qRT-PCR). TRAIL sensitivity was also determined for each cell line (sensitive = $IC_{50} \leq 50$ ng/ml, resistant = $IC_{50} > 50$ ng/ml). Results are reported in Table 1. Levels of the functional TRAIL receptors DR4 and DR5 were also evaluated as an internal control, since levels of these receptors are not expected to be associated with Six1 expression. Levels of DcR1 did not correlate with Six1 expression as expected and are not shown here. DcR2 was 58 ± 12 fg/ng 18s rRNA in cell lines with absent or below mean Six1 expression as opposed to 127 ± 36 fg/ng 8srRNA in cell lines with above mean Six1 expression ($p = 0.045$ t-test, mean Six1 level = 108 fg/ng 18s rRNA). Cell lines were also tested for TRAIL sensitivity by the MTT assay with sensitivity determined as $IC_{50} < 50$ ng/ml TRAIL. As expected, as Six1 levels were increased, cells were more likely

to be resistant to TRAIL (absent/below mean level Six1 versus above mean level Six1 as compared to TRAIL sensitive or not sensitive $p=0.03$ Fisher's Exact test).

Table 1. Endogenous Six1 expression correlates with TRAIL sensitivity and DcR2 expression. A. 15 well characterized cell lines were analyzed for Six1 expression by qRT-PCR (reported as absent/below mean versus above mean relative expression, mean expression = 108 fg/ng 18s rRNA), DcR2 expression (as fg/ng 18s rRNA and TRAIL sensitivity (sensitive = $IC_{50} \leq 50$ ng/ml). B. DR4, DR5 and DcR2 expression was measured in the 15 cell lines and compared to absent/below mean six1 expression versus above mean Six1 expression. Significantly greater DcR2 expression was associated with Six1 expression ($p=0.045$ t-test) while functional TRAIL receptor DR4 and DR5 levels were not significantly different.

A.

Cell Line	Description / Reference	DcR2 level (ag/ng 18s rRNA)	Six1 mRNA (absent/below or above mean)	TRAIL sensitive
OVCA 433	Serous Ov Ca / Bast, 1981	126	absent	Yes
OVCA 432	Serous Ov Ca / Bast, 1981	33	absent	No
OVCA 420	Serous Ov Ca / Bast, 1981	26	absent	No
OVCAR5	Ov Ca Ascites / Hamilton 1984	49	below	Yes
2008	Ov Ca / DeSaia, Orth 1994	96	below	Yes
DOV-13	Ov Ca / Duke University 1994	109	below	Yes
Snu251	Endometrioid Ov Ca / Yuan, 1997	13	below	Yes
OVCAR2	Ov Ca Ascites / Hamilton 1984	74	below	Yes
OV1847	Ov Ca / Hamilton, 1990	42	below	No
CaOV3	Ov Ca / ATCC, J. Fogh	21	below	Yes
PECOC167	Serous Ov Ca / Univ of CO , 2008	91	above	No
HeyC2	Serous Ov Ca, passed in mice	254	above	No
Hey	Serous Ov Ca/ Buick, 1985	161	above	No
SKOV3	Grade 2 Ov Ca / J. Fogh, 1977	72	above	No
A2780	Ov Ca / Hamilton, 1990	57	above	No

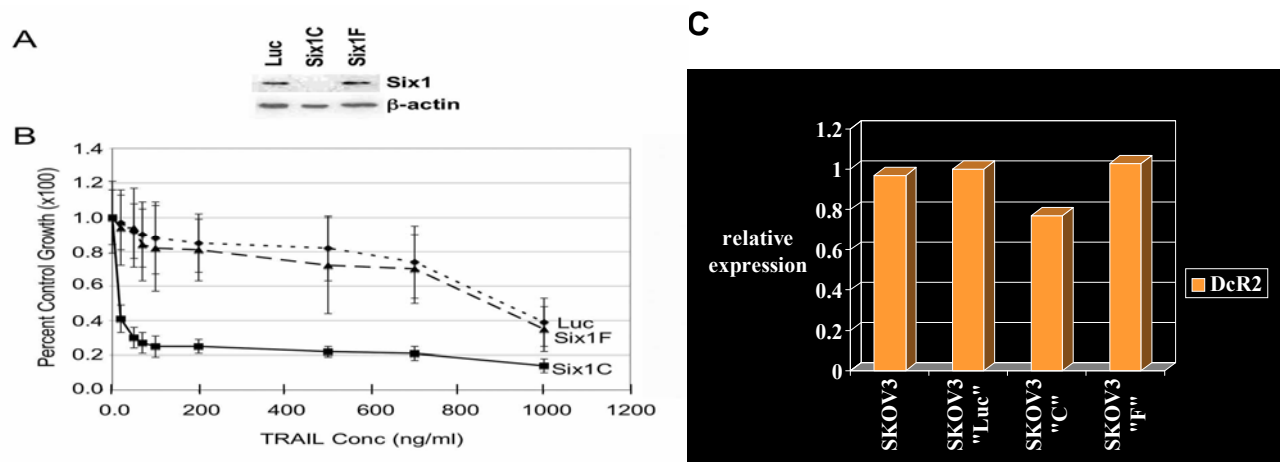
B.

Cell line Six1 Status	DR4 mRNA (ag/ng 18s rRNA)	DR5 mRNA (ag/ng 18s rRNA)	DcR2 mRNA (ag/ng 18s rRNA)
Absent or Below Mean	136±26	186±34	58±12
Above Mean	98±30	257±43	127±36
Significance	NS	NS	P=0.045

To perform a functional protein assay, cell surface expression of the DcR2 receptor was analyzed by flow cytometry using Phycoerythrin (PE) conjugated anti-DcR2 antibody (R&D Systems, Minneapolis, MN). Cell lines with absent or below mean Six1 levels demonstrated $5 \pm 2\%$ PE positive cells by flow cytometry as opposed to $43 \pm 25\%$ PE positive cells if Six1 was above the mean ($p=0.003$ t-test). Hence DcR2 is increased in Six1 overexpressing cells, both at the mRNA level and the functional cell surface expressed protein level.

With regard to studying over-expression in isogenic cell lines, CaOv3-Six1 over-expression studies showing associated DcR2 over-expression are reported in Figures 2 and 3 as justification for continuing studies on DcR2 as opposed to DcR1. SKOV3-siRNA knockdown studies were performed using SKOV3 siRNA active and sham constructs as reported in our published manuscript [8]. A Figure showing the effects of the control luciferase construct “luc”, the sham siRNA construct “F” and the active siRNA construct “C” on TRAIL dose-response from our manuscript is included below as Figures 5A-B. Figure 5A is a western blot for Six1 expression showing baseline high Six1 expression in the “luc” and sham “F” constructs and efficient Six1 knockdown in the “C” construct. Figure 5B shows the associated TRAIL sensitivity resulting from Six1 knockdown. Analysis of DcR2 by qRT-PCR from the parental SKOV3 cell line as well as from the constructs in Figure 5A is shown in Figure 5C. SKOV3 siRNA does cause decreased DcR2, but the magnitude of the decrease in DcR2 is less than what would be expected given the profound effect on TRAIL sensitivity in Figure 5A.

Figure 5. Effects of Six1 knockdown by siRNA in the SKOV3 cell line on TRAIL sensitivity and DcR2 expression. A. Western blot of Six1 with actin loading control shows efficient Six1 knockdown in the Six1 “C” construct but not the “luc” or Six1 “F” construct. B. Six1 knockdown sensitizes SKOV3 cells to TRAIL as evidenced by leftward shift of the dose-response in the Six1 “C” construct. C. DcR2 expression is decreased (relative expression 0.77) in the Six1 “C” construct as opposed to the parental SKOV3 cell line or the “luc” or Six1 “F” construct cell lines.



Hence task 1 is completed in year 2 as showing that DcR2 is upregulated in Six1 over-expressing cells and downregulated with Six1 knockdown.

Task 2. Determine whether *DcR2* is a direct or indirect target of Six1.

- a. Gel shift (electrophoretic mobility assay)
- b. Chromatin IP experiments
- c. Promoter activation studies

This task was initiated in year 2 focusing on DcR2 and was completed in the year three report.

To analyze the likelihood of DcR2 promoter binding by Six1, a 2000 bp sequence of the DcR2 mRNA upstream of the DcR2 translation start site was examined for the presence of the “TCAGG/CCTGA” consensus Six1 binding sequence[9] and 4 such sequences were found. Oligonucleotides (30 bp) of these regions were prepared and are listed in table 2. Results of the electrophoretic mobility assay are shown in Figure 6. An MEF3 site known to bind and gel-shift extracts of purified Six1 protein is shown as positive control. All the TCAGG sites in the DcR2 promoter region bound Six1 protein and shifted its movement on the gel, suggesting an interaction and supporting previously reported data.

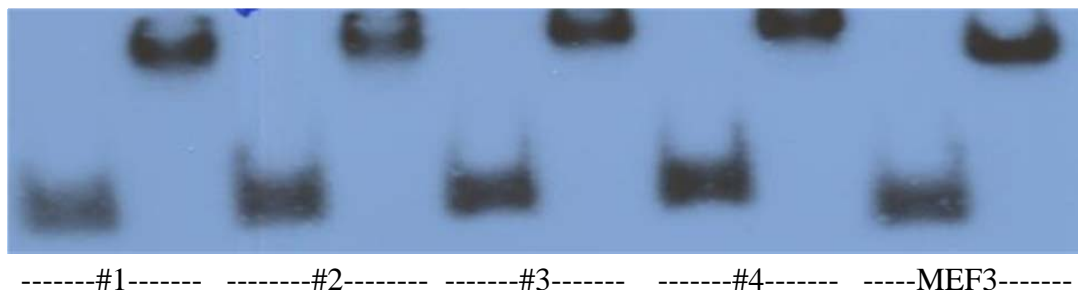


Figure 6. Gel-shift shows binding of 4 oligonucleotides containing consensus sites in DcR2 promoter to Six1 purified protein. Sequences for oligonucleotides 1-4 and MEF3 (consensus sequences in shaded box) are found in table. For each oligonucleotide, the first lane is the unbound negative control (probe) and the second lane is the bound oligonucleotide/protein complex showing the change in mobility. MEF3 is known to bind Six1 and is used as positive control.

Table 2. Oligonucleotide sequences for Figure 1.

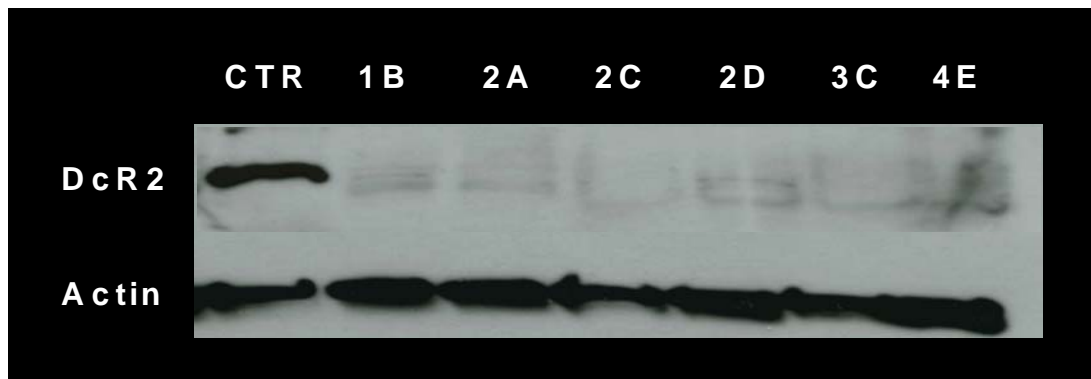
#1	TCA GTC TT	C CTG A	AG TC	C CTG A	CC TTT CAC (starting at bp 108)
#2	ACC ATG TGA GGG C	TC AGG	AGC CGA CTC ATC (starting at bp 1018)		
#3	GCC AGG AAG TAG T	TC AGG	GTT TAA GAA GAG (starting at bp 1624)		
#4	GGA GGG AGC AGG C	TC AGG	ATG GGC CTC CAG (starting at bp 1672)		
MEF3	GGG GGC	TCA GGT	TTC TGT GGC		

This data confirms our revised original hypothesis “The TRAIL decoy receptor DcR2 is a downstream target of Six1 in ovarian cancer cells”.

We also initiated DcR2 knockdown experiments to analyze if the same phenotype seen with Six1 knockdown could be recreated with DcR2 knockdown. This would give functional relevance to any Six1/DcR2 interaction we would find.

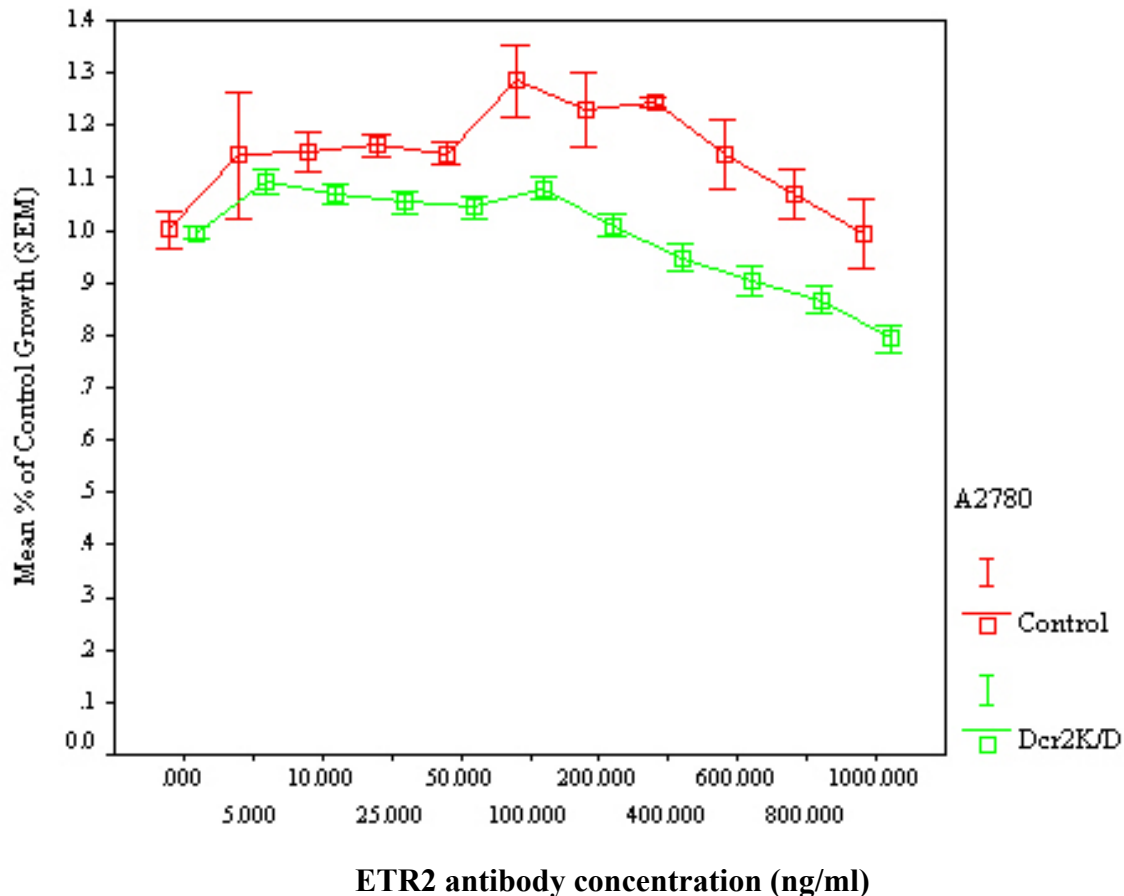
The A2780 cell line over-expresses Six1, has abundant DcR2, and is TRAIL and TRAIL agonistic antibody resistant. The SureSilencing (SABiosciences, Frederick, MD) shRNA knockdown system was used to generate multiple A2780 DcR2 knockdown cell lines using 4 different primer sets (numbered 1-4) and controls. A western blot of DcR2 with control CTR, 3 clones from primer set 2 (2A, 2C, and 2D) and a clone each from primer sets 1 (1B), 3 (3C) and 4 (4E) are shown in Figure 7. DcR2 was decreased in the knockdown clones as compared to the control clone.

Figure 7. DcR2 western blot of A2780 control clone (CTR) and knockdown clones (1B, 2A, 2C, 2D, 3C and 4E) with β -Actin loading controls.



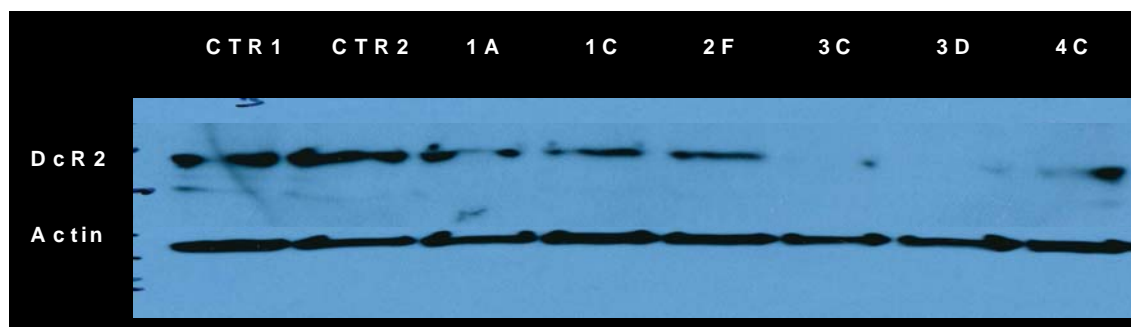
The effect of DcR2 knockdown on sensitivity to TRAIL and agonistic TRAIL antibodies was then studied by performing dose-response assays to TRAIL, FasL and ETR1 and ETR2 using control and DcR2 knockdown clones. Results are shown in Figure 8. DcR2 knockdown shifted the dose-response curve for ETR2 to the left in all cell lines (results show control versus 6 knockdown cell line curves pooled together), although the results were not as dramatic as that seen with Six1 knockdown in the SKOV3 cell lines (Figure 5B). This argues for additional mechanisms for Six1 dependent TRAIL resistance in ovarian cancer. There was no effect on TRAIL, FasL or ETR1 sensitivity (not shown).

Figure 8. DcR2 knockdown sensitizes A2780 cells to ETR2. Dose-response to the agnostic antibody to TRAIL-DR5, ETR2 are plotted as percent of control growth (\pm Standard Error of the Mean, S.E.M) for the A2780 CTR (control, upper line) cell line and the shRNA DcR2 knockdown clones 1B, 2A, 2C, 2D, 3C and 4E pooled together (Dcr2K/D, lower line). The two curves are significantly different ($p=0.02$ ANOVA)



In our previously published manuscript, Six1 knockdown sensitized SKOV3 cells to TRAIL. The SureSilencing shRNA knockdown system was also used to generate multiple SKOV3 DcR2 knockdown cell lines using 4 different primer sets (numbered 1-4) and controls. A western blot of DcR2 with controls CTR1 and CTR2, 2 clones from primer sets 1 (1A,1C) and 3 (3C,3D) and a clone from primer sets 2 (2F) and 4 (4C) are shown in Figure 9. DcR2 was decreased in the knockdown clones compared to controls.

Figure 9. DcR2 western blot of SKOV3 control clones (CTR1 and CTR2) and shRNA knockdown clones (1A,1C,2F,3C,3D and 4C) with β -Actin loading controls.



The effect of DcR2 knockdown on sensitivity to TRAIL and agonistic TRAIL antibodies was then studied by performing dose-response assays to TRAIL, FasL and ETR1 and ETR2 using control and DcR2 knockdown clones. DcR2 knockdown did not sensitize SKOV3 cells to TRAIL or ETR1 or ETR2. This is contrary to the slight sensitization to ETR2 seen with DcR2 knockdown in the A2780 cell lines (Figure 8). For comparison, results of the ETR2 dose response to SKOV3 DcR2 knockdown is shown in Figure 10.

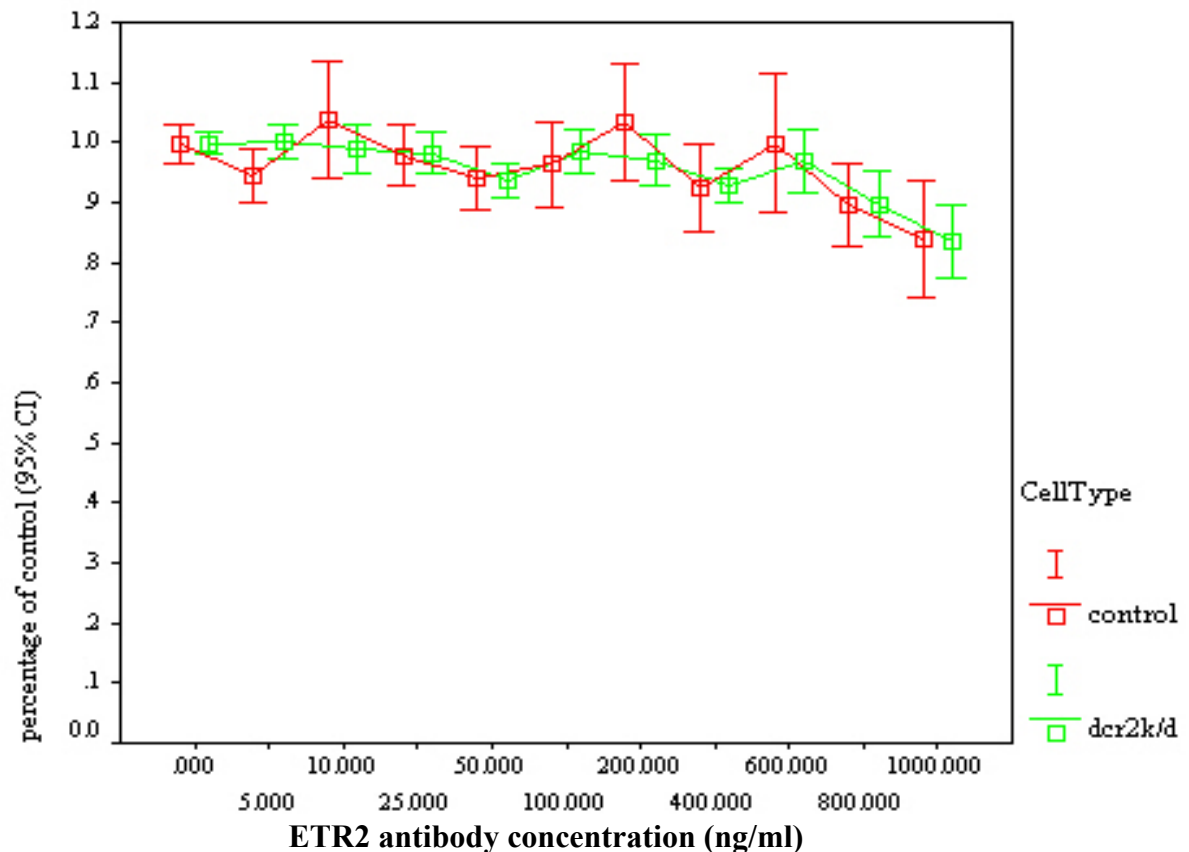


Figure 10. DcR2 knockdown does not sensitize SKOV3 cells to ETR2. Dose-response to the agonistic antibody to TRAIL-DR5, ETR2 are plotted as percent of control growth (\pm 95% Confidence Interval-CI) for the SKOV3 control cell line and the shRNA DcR2 knockdown clones 1A, 1C, 2F, 3C, 2D, 3D and 4C pooled together.

Overall, these studies show that DcR2 is a downstream target of Six1, but that manipulating DcR2 is not likely to have any uniform effect on the sensitivity of cells either to TRAIL or its receptor agonists. To further analyze the relationship between Six1 expression and TRAIL receptor expression, we studied TRAIL DR4, DR5, DcR1 and DcR2 cell surface receptor expression by flow cytometry in a panel of 15 ovarian cancer cell lines with Six1 levels ranging between 0 fg Six1/ng 18s rRNA to 763 fg Six1/ng 18s rRNA. Data is shown in Figure 11. As expected, increased Six1 correlated with increased DcR2 (Kruskal-Wallis test $p=0.05$), but not DcR1 or DR4. Interestingly, Six1 expression was also associated with increased DR5 (Kruskal-Wallis test $p=0.002$). Even though DcR2 was increased with increased Six1, the concomitant

DR5 increase was to the extent that the DR5/DcR2 ratio was also *greater* with increased DcR2 (we would hypothesize that it would be less, ie., more DcR2 as compared to DR5) implying that that the increase in DcR2 by Six1 may be compensated for by increased DR5 and that increased DcR2 may not be the mechanism for TRAIL resistance via receptor competition. Given this data, additional promoter activation studies and chromatin IP studies were not pursued. While this task is completed, additional analysis of TRAIL pathway components downstream of the receptors and the DISC complex in ovarian cancer, which may shed light on the Six1-mediated changes in the TRAIL pathway, and where manipulation of the pathway components can be used as therapy, is on-going in our laboratory.

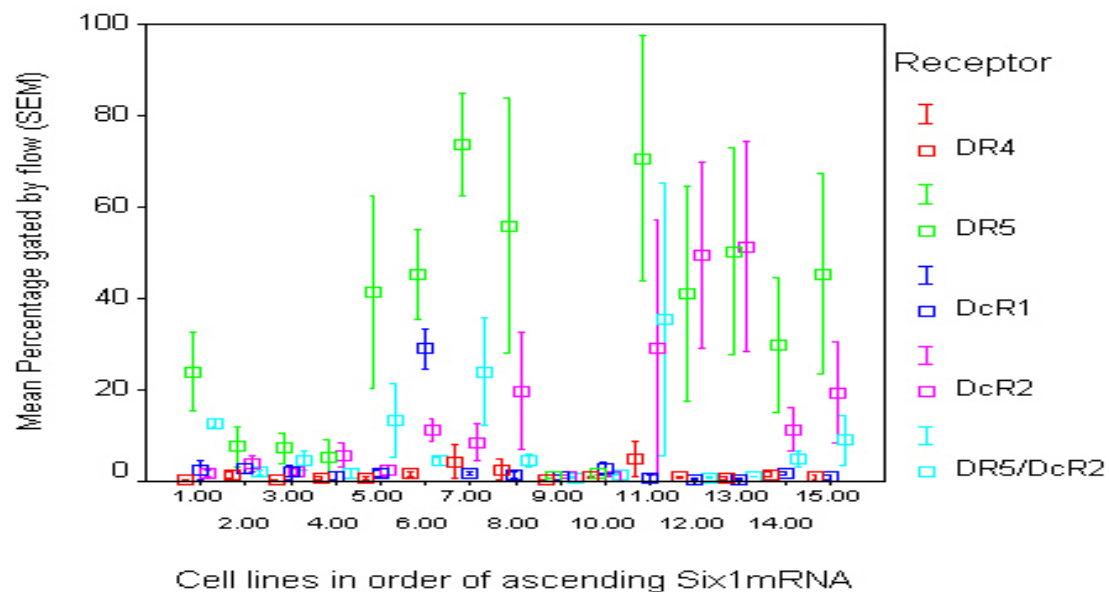


Figure 11. Analysis of TRAIL receptor level by flow cytometry in a panel of ovarian cancer cells with increasing levels of Six1. DcR2 mean percent positive cells by flow cytometry (\pm standard error of the mean SEM of 3-8 repeats) are low in the first five cell lines, but are increased in 8/10 subsequent cell lines. However, DR5 levels are similarly increased. The cell lines are 1. OV433 (0 fg Six1/ng 18srRNA), 2. OV432 (0 fg Six1/ng 18srRNA), 3. OV420 (0 fg Six1/ng 18srRNA), 4. OVCAR5 (5 fg Six1/ng 18srRNA), 5. OV2008 (17 fg Six1/ng 18srRNA), 6. DOV13 (19 fg Six1/ng 18srRNA), 7. SNU251 (36 fg Six1/ng 18srRNA), 8. OVCAR2 (81 fg Six1/ng 18srRNA), 9. OV1847 (85 fg Six1/ng 18srRNA), 10. CaOV3 (89 fg Six1/ng 18srRNA), 11. PECOC167 (109 fg Six1/ng 18srRNA), 12. HeyC2 (121 fg Six1/ng 18srRNA), 13. Hey (143 fg Six1/ng 18srRNA), 14. SKOV3 (155 fg Six1/ng 18srRNA), 15. A2780 (763 fg Six1/ng 18srRNA).

Task 3. Evaluate TRAIL panel sensitivity in Six1 over-expressing and knockdown cells.

- Generate inducible models of Six1 expression.
- Perform dose-response curves to TRAIL, FasL, ETR1, ETR2 using existing Six1-CaOV3 over-expression model and Six1 knockdown model, save cell pellets and extract RNA and protein.

In years 2 and 3, multiple systems were used to generate both inducible Six1 over-expression and inducible Six1 knockdown. These included the BD RevTet tetracyclin-on overexpression system and the P30ETREMIRAG lentiviral knockdown system. Neither yielded reproducible and tightly controlled effects as required for this task. While expected to have baseline levels of Six1, controls from the BD RevTet system also over-expressed Six1 suggesting that the system was either “leaky”, or the control media contained amounts of tetracycline sufficient to induce the transgene without the addition of any tetracycline. Certified tetracycline free media was ordered and the clones were re-isolated and grown. However, the transgene was expressed, even tetracycline-free media. The lentiviral knockdown system generated verified expression of the transgene as noted by the presence of a GFP tag on the selected clones, however, Six1 could not be suppressed.

To complete this task in the absence of an inducible model, additional stable CaOV3-Six1 over-expressing clones were generated using pcDNA3.1 plasmid transfection. A western blot for Six1 expression for the control (CAT-a1 and CAT-b1) and the Six1 overexpressing clones Six1-c1, Six1-c2, Six1-d1, Six1-d2 and Six1-e is shown in Figure 12 and shows successful stable Six1 expression in the transfected clones.

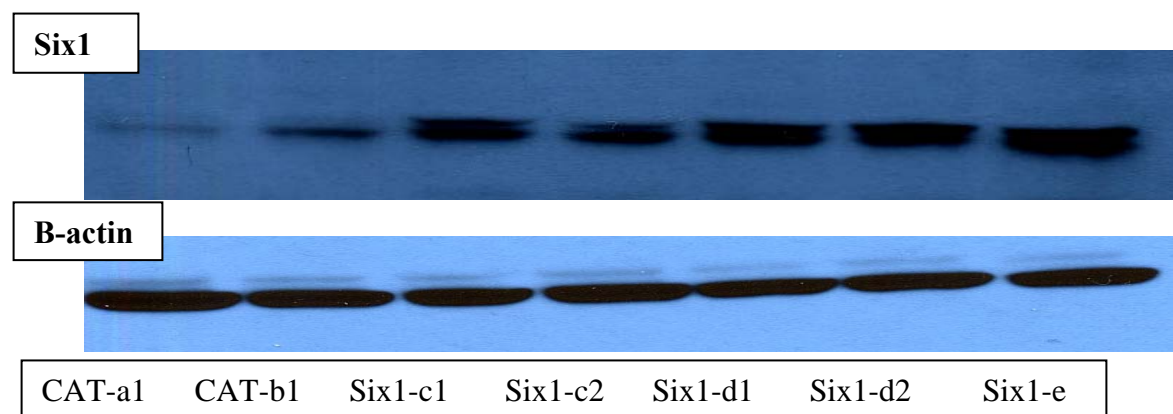


Figure 12. Generation of new CaOV3 Six1 over-expressing clones. A Western blot shows Six1 over-expression in the Six-c1, Six-c2, Six-d1, Six-d2 and Six-e clones as compared to the CaOV3 CAT-a1 and CAT-b1 clones.

TRAIL sensitivity was assayed in the clones above using by performing dose-response curves using the MTS assay. Six1 overexpression was confirmed to result in TRAIL resistance as shown in Figure 13. IC₅₀ values for Six1 over-expressing clones were 4-10 fold greater than in the CaOV3-CAT clones. This confirms our original hypothesis “Six1 overexpression in the CaOV3 ovarian cancer cell line blunts the response to TRAIL”.

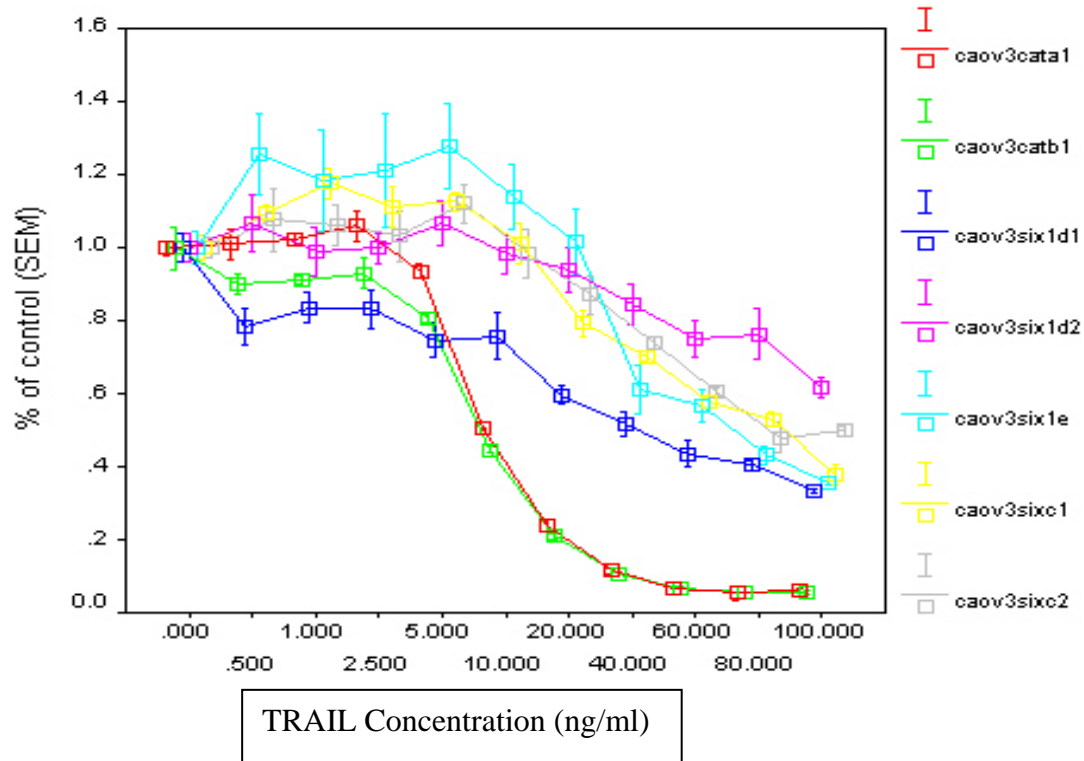


Figure 13. CaOV3 Six1 over-expressing clones develop resistance to TRAIL IC50 values are 10 ng/ml TRAIL for the CATa1 and CATb1 clones, 40 ng/ml TRAIL for the Six1-d1 clone, 80 ng/ml TRAIL for the Six1-c1, Six1-c2 and Six1-e clones and greater than 100 ng/ml TRAIL for the Six1-d2 clone.

ETR1 sensitivity was assayed in the clones above using by performing dose-response curves using the MTS assay. Results are presented in Figure 14. ETR1 sensitivity was seen in the CaOV3-CAT-a1 clone but not in the CaOV3-Catb1 clone or any of the CaOV3-Six1 clones. ETR2 sensitivity was assayed and showed a similar pattern (data not shown). The difference between TRAIL sensitivity and ETR1/ETR2 sensitivity in the CaOV3-CATb1 clone may be related to the slightly greater expression of Six1 in the CaOV3-b1 clone as compared to the CaOV3-CATa1 clone and implies a different threshold for TRAIL sensitivity as compared to TRAIL receptor agonist sensitivity. All clones were resistant to FasL with IC₅₀ > 5000 pg/ml.

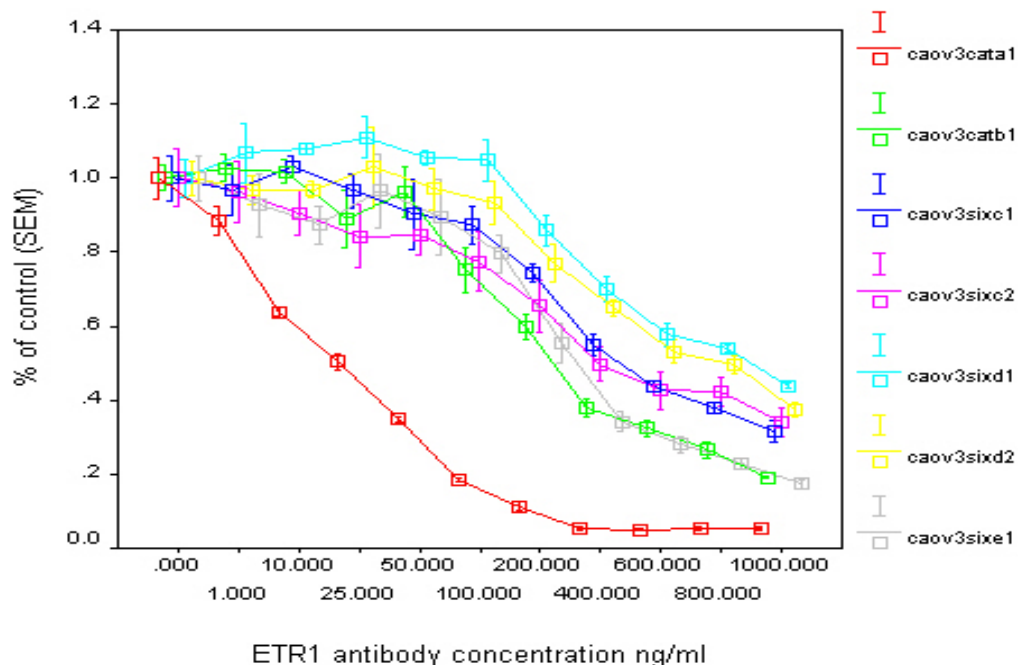


Figure 14. CaOV3 Six1 over-expressing clones develop resistance to ETR1. IC₅₀ values are 10 ng/ml ETR1 for the CATa1 clone, 200 ng/ml ETR1 for the CAT-b1 clone, 250-300 ng/ml ETR1 for the Six1-c1, Six1-c2 and Six1-e1 clone, and 900 ng/ml ETR1 for the Six1-d1 and Six1-d2 clones.

Preference was given to DcR2 knockdown experiments rather than repeat of the Six1 knockdown experiments after completion of tasks 1 and 2 revealed DcR2 to be a relevant target. Results of DcR2 knockdown experiments are reported under task 2.

Task 4. Evaluate TRAIL panel sensitivity in primary ovarian cancer cell lines and correlate with Six1 and DcR2 expression.

- Perform dose-response curves to TRAIL, FasL, ETR1, ETR2 using primary ovarian cancer cell lines, save cell pellets.
- Extract RNA and protein from cell pellets, correlate with Six1 and DcR2 expression

In year two, we reported that we had established cell lines from patients with ovarian cancer and tested sensitivity to TRAIL and TRAIL agonistic antibodies. Twenty-nine patients had been enrolled and 17 specimens had generated cell lines that could be assayed. All patient specimens were resistant to FasL up to 5000 pg/ml. 6/17 cell lines generated tumors in CB-17 SCID mice and tumorigenicity was associated with tumor Six1 expression ($p=0.02 \chi^2$). We reported no clear developing correlation between Six1 status and TRAIL resistance in primary cell lines derived from patient tumors, but subsequently discovered that some primary cell lines rapidly lose Six1 expression in culture and that the cells being tested for TRAIL sensitivity may not be similar to those growing in the patient. Hence we repeated the analysis using the established cell line Six1 mRNA level rather than the original tumor Six1 mRNA level. Results are presented in Table 3. No correlation was found between primary ovarian cancer cell line Six1 mRNA

expression (none versus any) and TRAIL or ETR2 resistance in primary cell lines generated from patients with metastatic ovarian cancer ($p=0.25$ χ^2 test).

Table 3. Primary cell lines isolated from patients with Age of the patient, Histology of the primary tumor, cell line Six1 level, tumorigenicity in CB-17 SCID mice and TRAIL, ETR1 and ETR2 IC₅₀. Resistance was defined as greater than 50 ng/ml for TRAIL and greater than 1000 pg/ml for ETR1 and ETR2. All cell lines were resistant to FasL up to 5000 pg/ml.

#	Age	Stage	Histology	Cell line Six1 fg/ng 18s rRNA	Tumors?	TRAIL IC ₅₀	ETR1 IC ₅₀	ETR2 IC ₅₀
141	75	IIIc	Serous	0	No	R	R	R
140	48	IIIc	Serous	0	No	R	R	R
137	58	IIIc	Serous	0	No	R	R	R
142	84	IV	Serous	0	No	R	R	R
139	56	IIIc	Serous	0	No	R	R	R
138	52	IIIc	Endo	0	Yes	R	R	R
163	52	IIIc	Serous	0	No	2.5 ng/ml	R	200 pg/ml
158	45	IIIc	Serous	1	Yes	5 ng/ml	R	600 pg/ml
162	57	IIIc	Serous	7	No	R	R	R
173	59	IIIb	Serous	7	Yes	R	R	R
150	65	IIIc	Serous	9	Yes	1 ng/ml	R	200 pg/ml
153	71	IV	Serous	24	No	R	R	R
161	52	IV	Serous	66	No	5 ng/ml	R	400 pg/ml
160	60	IV	Mucinous	179	No	R	R	R
167	47	IV	Mixed	220	Yes	R	R	R
159	43	IIIa	Clear Cell	311	No	R	R	R
164	52	IIIc	Serous	324	Yes	1 ng/ml	R	350 pg/ml

While these results did not support our hypothesis, we recognized that cell lines obtained from patient tumors may not be representative of the *in-vivo* tumor. Hence we have subsequently pursued a novel *ex-vivo* method to directly assay TRAIL sensitivity in relation to Six1 status in patient tumors. Preliminary experiments showed our ability to harvest tissues and process them using the Krumdieck tissue dissector (Alabama Research and Development), to maintain tissues *ex-vivo*, to study proliferation using standard MTS assay (CellTiter 96[®] AQueous Assay, Promega) in the *ex-vivo* system, and to assay proliferation, and detect apoptosis. To demonstrate ability to study proliferation, we treated 300 μ m slices of tumor from a 58 year old patient with type II (high grade serous) platinum sensitive recurrent ovarian tumor and omental metastases. A 2cm by 2 cm section of omental tumor was cored under sterile technique and sliced using the Krumdieck tissue slicer with sterile PBS in the flow chamber. Slices were transferred to 24 well

plates and covered with 4 ml of RPMI1640 media. After 24 hours, media was changed to control (media + vehicle) or cisplatin at 50 μ M or cisplatin + TNF-related apoptosis inducing ligand (TRAIL) at 50 ng/ml or TRAIL alone. After 3 hours, the MTS solution was added to the media for 4 hours. A 500 μ l aliquot was pipetted into each of 6 wells of a 96 well plate and read using an ELISA plate reader at 490 nm. Results (Figure 15) are reported as percent of media only controls. Six1 mRNA was assayed by qRT-PCR and revealed a low level of 2 ag Six1/fg 18s rRNA (SKOV3 reference 114 ag Six1/fg 18s rRNA). This low Six1 expressing tumor was sensitive to TRAIL as well as to Cisplatin.

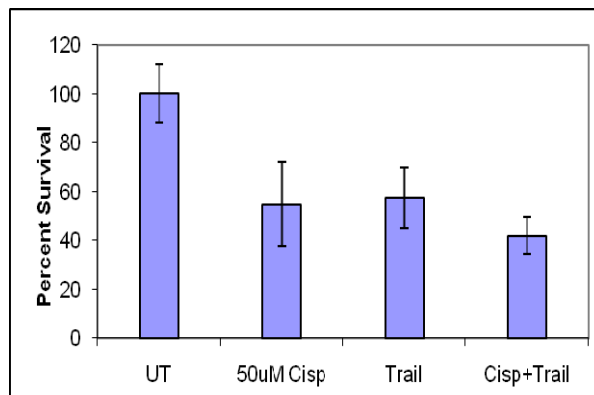


Figure 15. MTS assay from ex-vivo culture can detect proliferation differences. Tissue slices were treated with cisplatin, TRAIL, and cisplatin + TRAIL (reported as percent of control untreated-UT), 50 μ M cisplatin or 50 ng/ml TRAIL resulted in 60% of control proliferation. Addition of TRAIL to cisplatin did not decrease this further.

In the last year of the program, granted under the no-cost extension, a new antibody (Six1cTerm) was generated to the unique C-terminal region of Six1 and validated as detailed in the attached manuscript (submitted, April 2011). This antibody was used to correlate Six1 protein expression in the *ex-vivo* tissue slices with the MTS assay for TRAIL sensitivity. Six1 staining was assayed by a score multiplying staining intensity by percentage of stained epithelial cell nuclei as measured by two independent blinded observers (inter-observer correlation coefficient $\kappa = 0.76$, substantial). The *ex-vivo* slice was considered sensitive by MTS assay if inhibition as greater than 50% of control and statistically inhibited by the χ^2 test on triplicate measures. Mean age of the patient group was 61 yrs (range 52-72 years) and the majority had serous histology (6/11) with an additional 3 endometrioid cancers and 2 carcinosarcomas. Six1 homeoprotein was detected (score > 0) in 82% of the samples (9/11) and 8/11 were resistant to TRAIL by MTS assay. Six1 immunohistochemistry score was significantly associated with TRAIL resistance (Mann-Whintey U test $p = 0.048$)

Table 4. Comparison of Six1 protein expression in ex-vivo slices of omental tumor from (n=11) ovarian cancer patients with TRAIL sensitivity by MTS assay. Six1 homeoprotein expression predicts TRAIL resistance.

SAMPLE	SIX1 IHC	SIX1 IHC % positive	SIX1 Stain Intensity	SIX1 SCORE (% x intensity)	MTS FOR TRAIL
1	POS	10%	+1	10	Resistant
2	POS	60%	+3	180	Resistant
3	POS	10%	+3	30	Resistant
4	POS	25%	+2	50	Resistant
5	POS	5%	+1	5	Resistant
6	POS	50%	+3	150	Resistant
7	POS	35%	+3	105	Resistant
8	POS	70%	+3	270	Resistant
9	POS	15%	+1	15	Sensitive
10	NEG	NEG	NEG	0	Sensitive
11	NEG	NEG	NEG	0	Sensitive

This data confirms that Six1 expression in human ovarian cancers is also correlated with TRAIL resistance and completes Task 4 and 6.

Task 5. Establish Syngeneic (mouse) Six1 over-expression Model

Ten previously characterized mouse ovarian cancer cell lines [10] were obtained from Dr. Katherine Roby at the University of Kansas Medical Center under a 3 year Materials Transfer Agreement. MOSEC were tested for Six1 mRNA expression using mouse specific Six1 qRT-PCR primers and probes and results are shown in Table 5. Only the MOSEC IO8 cell line had any significant Six1 expression. Hence, Six1 expression is most likely not an important contributor to mouse ovarian cancers and given our development of *ex-vivo* models for studying primary human ovarian tumors, development of a syngeneic mouse model was not pursued.

Table 5. Six1 expression in MOSEC cell lines. Only the IO8 cell line had any significant expression of Six1. Results are reported as fg/ng 18s rRNA.

Cell line	Six1 expression (fg/ng 18s rRNA)
MOSEC 2C6	3
MOSEC 2C12	0
MOSEC ID9	0
MOSEC IO8	43
MOSEC ID5	16
MOSEC IF5	5
MOSEC IC5	0
MOSEC 3B11	0
MOSEC 3E3	1
MOSEC IG10	0

Task 6. Xenograft and/or syngeneic model Six1/DcR2 over-expression and knockdown analysis.

- Test TRAIL, Etoposide (instead of FasL), ETR1 and ETR2 response in xenograft or syngeneic model.
- Evaluate phenotype of in-vivo Six1/DcR2 knockdown

For this final report, this task was incorporated into task 4 based on data presented in the introduction. Data is presented under task 4.

KEY RESEARCH ACCOMPLISHMENTS:

- Six1 overexpression is associated with TRAIL resistance both *in-vitro* and in patient tumor specimens *ex-vivo*.
- Overexpression of the TRAIL DcR2 decoy receptor in ovarian cancer cell lines and in a syngeneic over-expression system is correlated with Six1 over-expression. However, manipulation of DcR2 does not seem to affect TRAIL sensitivity, raising the possibility that Six1 may affect other TRAIL pathway components.
- Ovarian cancer cells express TRAIL DR4, DR5 and the decoy receptor DcR2. DcR1 expression is uncommon.
- Tumorigenicity in ovarian cancer cell lines and in Six1 over-expression and knockdown models is related to Six1 expression.

REPORTABLE OUTCOMES :

The following manuscripts have been published in peer reviewed journals as a result of this research (pdf attached):

Menke C, Goncharov T, Qamar L, Korch C, Ford HL, **Behbakht K**, and Thorburn A. TRAIL Receptor signaling regulation of chemosensitivity in vivo but not in vitro. PLoS ONE. 14:6(1):e14527, Jan 2011.

The following manuscripts have been submitted to peer reviewed journals as a result of this research (pdf attached):

Qamar L, Deitsch E, Patrick AN, Post MD, Spillman MA, Thorburn A, Ford HL, **Behbakht K**. Development and Prognostic Validation of a Polyclonal Antibody to Detect Six1 Homeoprotein in Ovarian Cancer. Submitted – Molecular Oncology, April 2011.

The following manuscripts are under preparation as a result of this research:

Embry J, Qamar L, Davidson SA, Spillman MA, Behbakht K. Overexpression of the Six1 homeobox gene affects the metastatic patterns of stage III and IV ovarian tumors.

Anderson CK, Qamar L, Spillman MC, Post MD, Thorburn A, Ford FL and Behbakht K. Homeobox Six1 Expression and TRAIL receptor Expression in Ovarian Cancers.

The following abstracts were presented at a national meeting as a result of this research (pdfs attached):

Qamar L, Syed N, Ford H, Thorburn A, Behbakht K. The Six1 homeobox gene is associated with TRAIL resistance in ovarian cancer and is correlated with increased Tumor Necrosis Factor-Related Apoptosis Inducing Ligand (TRAIL) decoy receptor DcR2 in a Six1 overexpression model. Presented at the 40th Annual Meeting of the Society of Gynecologic Oncologists, published *Gynecol Oncol* 112 (2009) page S158.

Anderson CK, Qamar L, Davidson SA, Spillman MA, Behbakht K. Using an ex-vivo tumor slice system to investigate experimental therapies in epithelial ovarian carcinoma. Presented at the Western Association of Gynecologic Oncologists, June 2011.

CONCLUSIONS:

Overexpression of the developmental homeobox gene Six1 is gaining importance as a mechanism for carcinogenesis and metastasis in an ever-growing list of malignancies. The list of downstream genes controlled by Six1 is also ever-growing and likely to be tissue specific. We

have discovered that overexpression of the Six1 homeobox gene in ovarian cancer is associated with TRAIL resistance, but even though the TRAIL decoy receptor DcR2 is a downstream target of Six1, manipulation of DcR2 is unlikely to have therapeutic impact. We plan to conclude further mechanistic and functional studies via an NIH-R01 (CA124545, triple P.I. Thorburn, Behbakht and Ford) that has been funded with preliminary data justification supported by this award. Submission for a Teal Expansion Award is planned.

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TRAIL Receptor Signaling Regulation of Chemosensitivity In Vivo but Not In Vitro

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Abstract

Background: Signaling by Tumor Necrosis Factor-Related Apoptosis Inducing Ligand (TRAIL) and Fas ligand (FasL) has been proposed to contribute to the chemosensitivity of tumor cells treated with various other anti-cancer agents. However, the importance of these effects and whether there are differences in vitro and in vivo is unclear.

Methodology/Principal Findings: To assess the relative contribution of death receptor pathways to this sensitivity and to determine whether these effects are intrinsic to the tumor cells, we compared the chemosensitivity of isogenic BJAB human lymphoma cells where Fas and TRAIL receptors or just TRAIL receptors were inhibited using mutants of the adaptor protein FADD or by altering the expression of the homeobox transcription factor Six1. Inhibition of TRAIL receptors did not affect in vitro tumor cell killing by various anti-cancer agents indicating that chemosensitivity is not significantly affected by the tumor cell-intrinsic activation of death receptor signaling. However, selective inhibition of TRAIL receptor signaling caused reduced tumor regression and clearance in vivo when tested in a NOD/SCID mouse model.

Conclusions: These data show that TRAIL receptor signaling in tumor cells can determine chemosensitivity in vivo but not in vitro and thus imply that TRAIL resistance makes tumors less susceptible to conventional cytotoxic anti-cancer drugs as well as drugs that directly target the TRAIL receptors.

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Introduction

The death receptors DR4 and DR5 activate signaling and apoptosis in response to the Tumor Necrosis factor-Related Apoptosis-Inducing Ligand (TRAIL), while Fas/CD95 activates apoptosis in response to Fas ligand (FasL). These receptors are the main executioners of the “extrinsic” apoptosis pathway that activate the apoptosis machinery by forming a complex called the Death Inducing Signaling Complex (DISC). The DISC is formed when a ligand-bound receptor complex recruits the adaptor protein FADD, which leads to the recruitment, dimerization [1], and catalytic activation of caspase-8 [2–4]. Active caspase-8 directly activates the effector caspase-3 and stimulates the mitochondrial (intrinsic) apoptosis pathway by cleaving the BH3 protein Bid. This allows Bid’s translocation in to the mitochondria and Bax/Bak-dependent release of cytochrome c and other pro-apoptotic proteins, with subsequent amplification of effector caspase activity. There is considerable interest in targeting the TRAIL receptors using pro-apoptotic receptor agonists [2] and clinical trials using recombinant TRAIL and antibodies that target DR5 or DR4 are underway.

The TRAIL and Fas pathways are important in anti-tumor and anti-metastasis responses mediated through the immune system [3,5]. TRAIL signaling mediates T-cell- and natural killer (NK) cell-dependent metastasis suppression in xenografts [6–9]. Autochthonous models show that deficiency in TRAIL receptor signaling promotes tumorigenesis [10] and metastasis [11]. Fas signaling has also been proposed as a mechanism by which NK cells can eliminate tumor cells [12]. Conversely, Fas signaling can also be a mechanism by which tumors counteract immune-mediated anti-tumor responses [13]. Moreover, both Fas [14] and TRAIL [15] have non-apoptotic signaling activities that promote tumor progression if the apoptotic response is blocked. Tumor cells can become resistant to death receptor signaling through multiple mechanisms [16]. Some of these mechanisms e.g. down-regulation of FADD [17] or increased expression of the caspase-8-like protein FLIP [18] affect both Fas and TRAIL receptors whereas, other mechanisms are more selective. For example, somatic mutations in DR5 cause a dominant negative phenotype that blocks TRAIL signaling through DR4 and DR5, but has no effect on Fas signaling [19]. Similarly, increased expression of the

homeobox transcription factor Six1 is a common tumor defect that arises in the majority of patients with metastatic ovarian or breast cancer, is associated with poor clinical outcome in multiple tumor types [20] and causes inhibition of TRAIL but not FasL-induced apoptosis [21].

Most anti-cancer drugs function by activating the mitochondrial apoptosis pathway; however, it has been suggested that death receptor signaling also contributes to the overall anti-tumor response to diverse chemotherapeutic drugs. Drug and radiation induced killing of brain tumor [22] and hepatoma [23] cells have been reported to rely on Fas signaling. Experiments, where TRAIL signaling was inhibited by silencing DR5 [24] or by increasing the expression of the decoy receptor DcR2 [25], led to the conclusion that chemosensitivity to 5-fluorouracil, doxorubicin, and etoposide depends on TRAIL receptor signaling. These effects have been demonstrated *in vitro* with cell lines, suggesting they are intrinsic to tumor cells. These effects can also be achieved by increased expression of death receptors and/or ligands that create a tumor cell-intrinsic autocrine signaling loop. Similar mechanisms of death receptor up-regulation have been proposed as an explanation for how various cytotoxic chemotherapeutic agents synergize TRAIL receptor-targeted agonists [26].

However, it is unclear if the Fas and TRAIL receptor pathways are really important contributors to tumor chemosensitivity. Since the activation of the mitochondrial apoptosis pathway leads to efficient cell killing, one would expect that drugs that are able to activate the mitochondrial pathway (i.e., most anti-cancer agents) should not require additional death receptor signaling in order to die, unless the pro-apoptotic signal from the mitochondria was insufficient to force the cell to cross its apoptotic threshold. It is less clear if the same considerations apply *in vivo*, where other inputs (e.g., from other cell types) may play a role. To address this question, we constructed isogenic tumor cell lines that are functional for both TRAIL and FasL signaling, inhibited for both or inhibited for just TRAIL signaling. We show that even in a cell line in which blocking death receptor-induced apoptosis has no detectable effect on the sensitivity to various chemotherapeutic agents and other apoptotic inducers *in vitro*, inhibition of TRAIL receptor signaling *in vivo* affects sensitivity to an anti-cancer drug. These data indicate that the presence of a functional TRAIL receptor apoptosis pathway can regulate chemosensitivity through tumor cell extrinsic mechanisms.

Results and Discussion

Selective inhibition of death receptor signaling with FADD-DD mutants

FADD is required for both TRAIL- and FasL-induced apoptosis. One way signaling can be inhibited by these receptors is by overexpressing a version of FADD (FADD-DD) that contains the FADD death domain, but lacks the death effector domain that binds to caspase-8. This molecule has been thought to inhibit signaling by competing with endogenous FADD protein for binding to the activated death receptors. However, based on data showing that FADD must self-associate via its death effector domain in order to bind to death receptors, it has been proposed that the isolated FADD death domain should be unable to bind to or efficiently inhibit Fas signaling [27]. Therefore, we first tested if we could obtain effective and selective inhibition of death receptor-induced apoptosis using FADD-DD and FADD-DD V108E, a mutant that was selected for its inability to bind to Fas, while retaining the ability to bind to TRAIL receptors [28]. Dose response curves (Figure 1A) using FasL or TRAIL with three isogenic BJAB cell lines expressing, GFP, GFP-FADD-DD or GFP-FADD-DD (V108E) showed that FADD-DD and FADD-

DD (V108E) effectively inhibited apoptosis induced by TRAIL and agonistic TRAIL receptor antibodies. However, only the wildtype FADD-DD molecule inhibited FasL-induced death.

To test if inhibition of receptor-induced apoptosis was due to binding of the FADD-DD molecules to the activated receptors, we performed DISC immunoprecipitation experiments (Fig. 1B). Upon activation of the receptor, FADD-DD was recruited to both Fas and TRAIL receptors instead of the endogenous FADD protein, which was recruited in the control cells. The V108E mutant was recruited only to activated TRAIL receptors. These data indicate that FADD-DD molecules are effective inhibitors of death receptor signaling and that their mechanism of action is through recruitment to the activated receptor in place of endogenous FADD protein. However, because the level of the FADD-DD mutants (Fig. 1A) in the cells is about 200-fold higher than the endogenous FADD protein, while the amount of the FADD-DD recruited to activated receptors is similar to the amount of endogenous FADD that is recruited, our data are consistent with the conclusion of Sandu et al. [27] that the isolated death domain is less efficiently recruited to the receptors compared with the endogenous protein. Fig. 1C demonstrates that the FADD-DD molecule also blocks both FasL and TRAIL-induced activation of downstream kinase pathways activating JNK and causes degradation of I κ B. The V108E mutant only affects TRAIL-induced activation of these pathways, which are known to be activated in a FADD-dependent manner [29].

Inhibition of Fas and TRAIL receptor-induced apoptosis has no effect on the efficiency of tumor cell killing by diverse chemotherapeutic agents and apoptotic stimuli *in vitro*

To test whether death receptor signaling alters the sensitivity of tumor cells to other agents, we assessed dose response curves for the three isogenic cell lines with agents that work by different mechanisms. Overlapping dose response curves (Fig. 2) showed that they had no measurable effect on tumor cell killing by various types of agents that target activities that are relevant for anti-cancer treatment. We observed this for a topoisomerase inhibitor (etoposide), histone deacetylase inhibitors (oxamflatin, MS275), an anthracycline (doxorubicin), a proteasome inhibitor (MG132), DNA damaging agents (UV, temozolomide) and an antimetabolite (5-fluorouracil). Similarly, there was no effect of FADD-DD or the V108E mutant on tumor cell killing by general apoptotic stimuli including the broad-spectrum protein kinase inhibitor staurosporine and increased hyperosmolar stress (sorbitol). MTS assays assess cell viability over a relatively short term and thus are not truly comparable to long-term tumor growth responses *in vivo*. To ensure that the selective inhibition of TRAIL-induced death without affecting survival in response to cytotoxic chemotherapy affected long-term growth, we performed a cell grow back assay by treating cells for 24 hours with TRAIL or etoposide then washing out the drug and allowing any surviving cells to grow back. Figure 3 shows that the TRAIL-treated FADD-DD expressing cells displayed equivalent growth over 7 days to untreated cells whereas the same cells died in response to etoposide treatment. Thus even with a more rigorous tumor cell survival assay where any surviving cells had several days to recover and grow in the absence of drug, FADD-DD provides no protection against etoposide-induced death, while providing complete protection against TRAIL.

These data run counter to some other studies. For example, Liu et al. [25] concluded that increased expression of DcR2, which is a decoy receptor that selectively inhibits TRAIL signaling, reduced *in vitro* chemosensitivity to doxorubicin and etoposide, while Wang

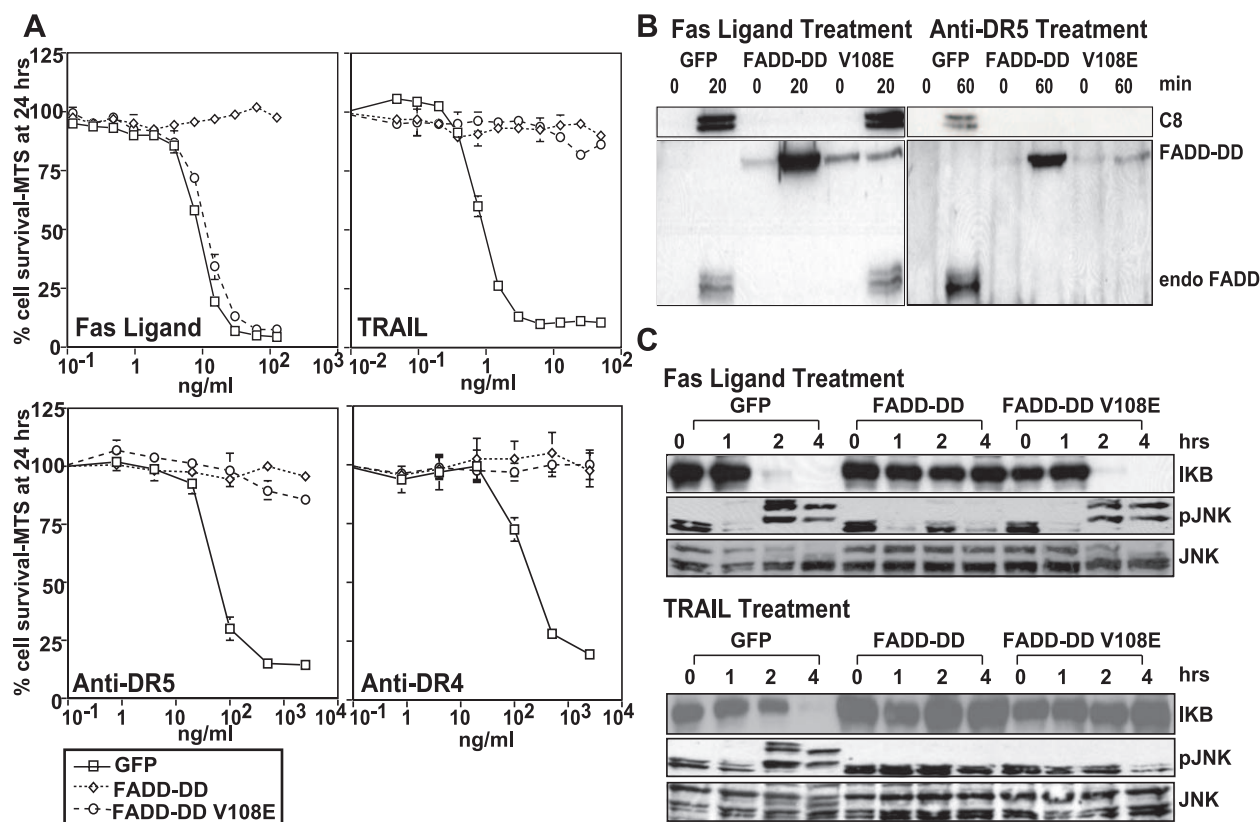


Figure 1. Selective inhibition of FasL and TRAIL or TRAIL-induced signaling and apoptosis. In panel A, isogenic BJAB cells expressing GFP or GFP-FADD-DD and FADD-DDV108E were treated with increasing doses of Fas ligand, TRAIL, or agonistic anti-DR4 and anti-DR5 antibodies. The differences in responses indicate that both FADD-DD expressing cell lines were resistant to all TRAIL R targeted drugs, but that only FADD-DD expressing cells are resistant to FasL. In panel B, DISC IP experiments precipitating Fas or DR5 followed by western blotting for casp-8 or FADD demonstrate an increased recruitment of FADD-DD in place of endogenous FADD to the receptors. Panel C, illustrates that on activation of other signaling pathways leading to I κ B degradation, and JNK phosphorylation, FADD-DD blocks signaling. This block leads to these events for both FasL and TRAIL, and that FADD-DD V108E blocks signaling only for TRAIL.
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and El-Deiry concluded that knockdown of the TRAIL receptor DR5 conferred resistance to 5-fluorouracil [24]. We therefore repeated our studies in a colon cancer cell line that was used by other investigators who reported effects on chemosensitivity. Figure 4 shows that HCT116 cells expressing FADD-DD were resistant to both TRAIL and FasL, while the FADD-DD (V108E) expressing cells were resistant only to TRAIL. However, neither of these cell lines displayed significantly increased resistance to 5-fluorouracil, etoposide, or doxorubicin. Additionally, we determined whether combination treatments with TRAIL and other anti-cancer agents demonstrated a requirement for death receptor signaling for optimal activity of the other drug. Combination treatments using TRAIL with 5-FU, Doxorubicin, or etoposide all showed increased tumor killing compared with treatment with the cytotoxic agent alone. However FADD-DD or FADD-DDV108E expression only blocked the component of the death due to the death receptor agonist (data not shown). These data indicate that tumor cell intrinsic signaling through the Fas and TRAIL receptors does not significantly contribute to the killing activity of the other stimuli in BJAB cells or in HCT-116 cells.

Inhibition of TRAIL receptor-induced apoptosis promotes tumor growth and confers chemoresistance in vivo

We next tested whether the FADD-DD constructs conferred an effect in vivo by growing xenograft tumors with each of the

isogenic BJAB cell lines and treating with one of the agents (etoposide) that had no effect in vitro. Figure 5 shows that etoposide treatment caused almost complete tumor regression for the wildtype BJAB cells; whereas, the cells expressing FADD-DD or FADD-DD (V108E) displayed significantly less tumor regression ($p < 0.05$) by etoposide. These data indicate that the same tumor cells whose sensitivity to etoposide is not affected by FADD-DD or the V108E mutant in vitro (in both short- and long-term assays), do display reduced chemosensitivity in vivo. Western blotting of tumor tissue showed that the tumors retained similar levels of expression of the GFP-tagged proteins in each case. This shows that the death receptor-dependent aspect of etoposide function is achieved through a tumor cell extrinsic mechanism. Because the FADD-DD and FADD-DD V108E mutants were equally effective at blocking tumor regression caused by etoposide, we conclude that signaling through TRAIL receptors alone is sufficient to cause these effects.

The FADD-DD mutant is a useful tool, because it is highly specific and effective; however, such dominant negatives have not been found in human tumors. Therefore, to test whether these effects also apply when tumor cells are resistant to TRAIL through a mechanism that is relevant in human tumors, we compared isogenic BJAB cells that do or do not express the homeobox transcription factor Six1. Previously, we have shown that Six1 confers TRAIL resistance, but has little effect on FasL sensitivity

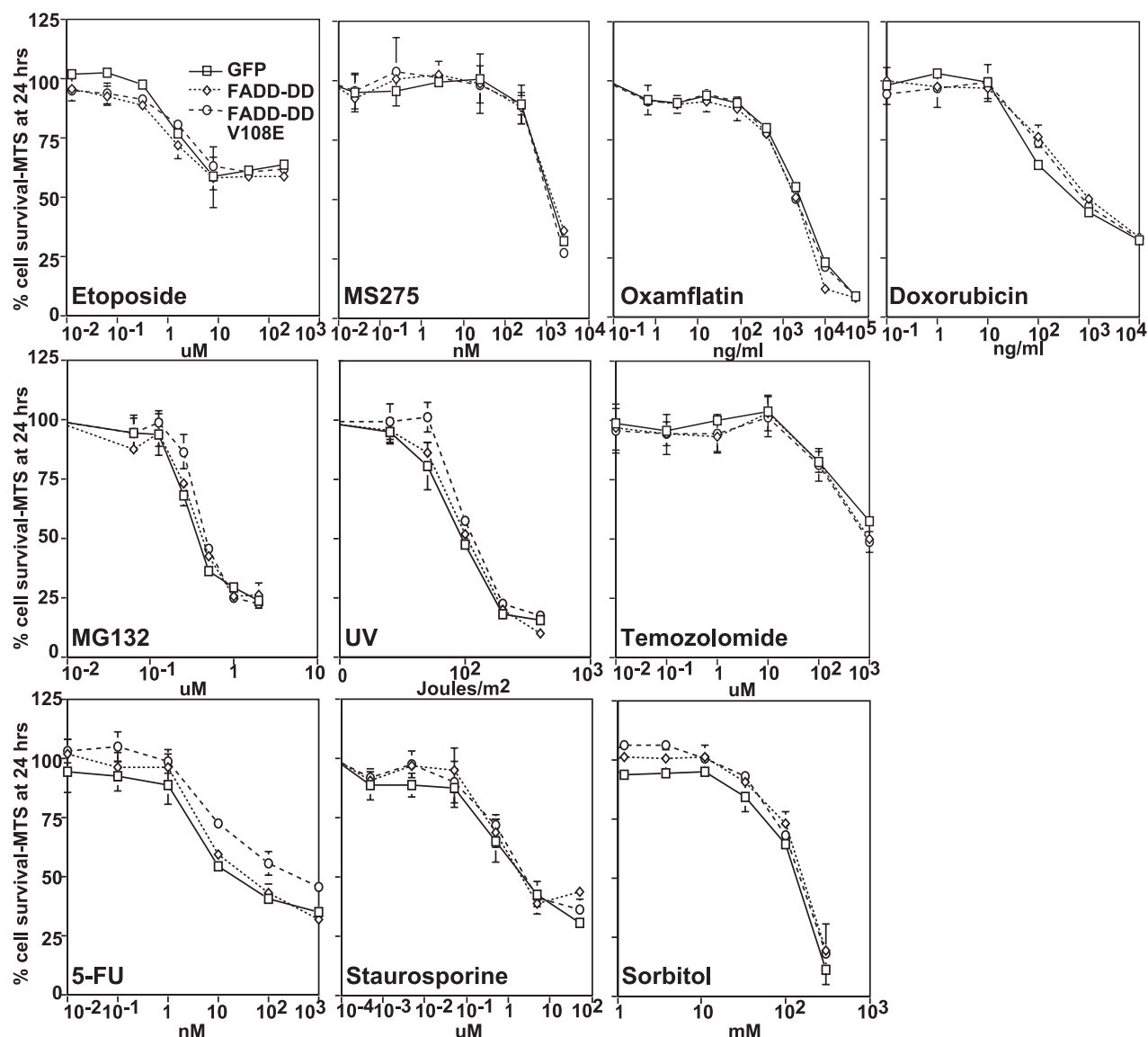


Figure 2. FADD-DD and FADD-DD V108E do not inhibit killing by other apoptotic stimuli. Isogenic BJAB cell lines were treated with increasing doses of etoposide, MS-275, oxamflatin, doxorubicin, MG132, UV, temozolomide, 5-FU, staurosporine or sorbitol as indicated followed by MTT assay to assess cell viability. All dose response curves overlap for each stimulus. doi:10.1371/journal.pone.0014527.g002

[21]. Six1-expressing BJAB cells are TRAIL-resistant (Fig. 6A) to a somewhat lesser degree than FADD-DD expressing cells (Fig. 1). However, these Six1-expressing cells do not demonstrate altered FasL or etoposide sensitivity in vitro. Furthermore, these cells displayed reduced chemosensitivity to etoposide ($p < 0.05$) when tested in vivo (Fig. 6B). Thus, the in vivo dependence of TRAIL receptor signaling for maximal chemosensitivity to another drug like etoposide also applies for a TRAIL resistance mechanism that is commonly found in human tumors and associated with poor clinical outcomes.

Conclusions

Using isogenic tumor cells that differ only in their ability to undergo apoptosis in response to Fas or TRAIL receptor activation, we found that various anti-cancer agents display no significant difference in their ability to be killed in vitro by anti-cancer drugs. This shows that, in general, cancer chemotherapy

drugs do not need to work through the death receptors. However, our results demonstrate a quite different and surprising result in vivo; etoposide, which was unaffected in vitro by TRAIL receptor or TRAIL and Fas receptor inhibition was significantly less effective in vivo and was unable to cause regression of these tumors. Instead, treatment led to stable tumor size when death receptor signaling was inhibited in the tumor cells. The lack of correlation between the in vitro and in vivo experiments carried out with the same cells indicates that even in tumor cells where activation of TRAIL receptors is not an important component of tumor cell killing in response to chemotherapy, tumor regression and clearance after treatment with a DNA damaging agent requires TRAIL receptor signaling.

This work suggests that tumors, which have evolved TRAIL resistance mechanisms [16] such as Six1 overexpression will not only respond less well to drugs such as Apo2L/TRAIL, lexatimumab, mapatumumab, ApoMab, AMG 655 etc. [2] that directly

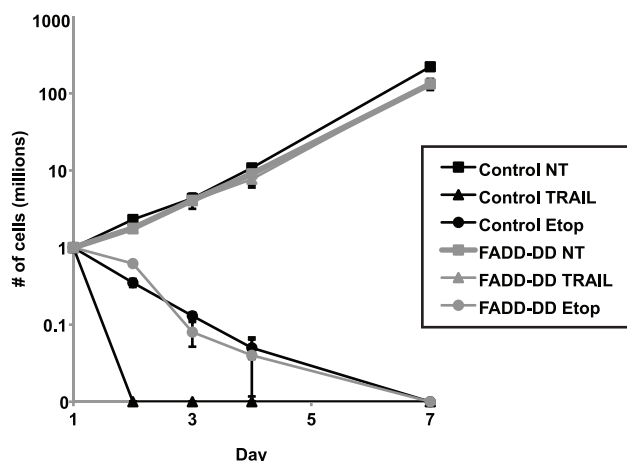


Figure 3. FADD-DD blocks TRAIL-induced but not etoposide-induced death in long-term assays. Isogenic control or FADD-DD expressing BJAB cells were treated with TRAIL or etoposide as indicated for 24 hours, then washed and replaced into growth media. Long term growth of surviving cells was determined by counting viable cells. Control BJAB cells died rapidly and were unable to recover any long term growth. Etoposide treated cells were completely unable to recover growth capacity whether or not FADD-DD was expressed. However FADD-DD expression protected the TRAIL-treated cells as demonstrated by overlapping growth curves with the untreated controls. doi:10.1371/journal.pone.0014527.g003

activate TRAIL receptors, but may also respond less well in vivo to conventional cytotoxic chemotherapy. The tumors were grown in NOD/SCID mice that lack T cells, but not Natural Killer cells or macrophages, suggesting that these cells are most likely the source of the TRAIL signal. Recent work has demonstrated the importance of the adaptive immune system, especially T cells, to the overall effectiveness of cancer chemotherapy (for review see [30]). Our data suggest that immune cell-mediated mechanisms working through TRAIL contribute to efficient tumor clearance after cytotoxic chemotherapy even without T cell involvement and these effects may add to any T cell mediated tumor clearance occurring after chemotherapy treatment. These data suggest that efforts to bypass TRAIL resistance would improve the efficacy of chemotherapy as well as improving the usefulness of drugs that are specifically targeted to TRAIL receptors.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee of the University of Colorado, Anschutz Medical Campus (protocol # 72609(12)1E).

Cell Lines

Parental BJAB cells were described previously [19], the various resistant cells expressing FADD-DD and FADD-DD V108E were made by stably expressing the respective cDNAs in pcDNA3.1 puro(+)-GFP. Six1-expressing cells were made by stably expressing the cDNA in pcDNA3.1 puro (+). All cell clones were derived from representative single clones isolated by limiting dilution. Cells were grown in RPMI 1640 with 10% FBS, sodium bicarbonate, and glucose in a 5% CO₂ humidified atmosphere at 37°C. The BJAB

cells used in these studies were DNA profiled using the Identifier kit (Applied Biosystems) in January 2010. We have not found any publication of a DNA profile for BJAB cells, nor are there any profiles for these cells or ones that have a matching profile in our own database (CK) or in publicly available databases. These include the consolidation of the DSMZ, ATCC, JCRB, and Riken databases of DNA profiles of cell lines now available at DSMZ website (www.DSMZ.de). Therefore, it is impossible to compare our sample of these cells to samples of this line used in other reports. However, this analysis did exclude contamination with any common cell lines that have been previously profiled. The profile we obtained for these cells is the following: Amelogenin: X; CSF1PO: 8,10; D2S1338: 18, 21; D3S1358: 16; D5S818: 12, 13; D7S820: 10, 11; D8S1179: 14, 15; D13S317: 9,11; D16S539: 9, 11; D18S51: 16, 22; D19S433: 12, 14; D21S11: 27, 28; FGA: 27, 28; TH01: 7; TPOX: 6,9; vWA: 14, 16.

DISC IP

Fas Ligand— 2.5×10^7 cells were suspended in 25 ml of culture medium, incubated with SuperFasLigand (Enzo Life Sciences, Plymouth Meeting, PA) at 1.25 μ g/ml at 37°C for 20 min, washed in phosphate-buffered saline three times, and then lysed in IP buffer (150 mM NaCl, 25 mM Tris·Cl, pH 7.5/1% Triton X-100, 4 mM EDTA) supplemented with complete protease inhibitors (Roche Applied Science) for 1 hr at 4°C. After the lysates were centrifuged (15 min at 13,000 rpm), lysates were precleared for 1 hr at 4°C with Glutathione-Agarose beads (Sigma, St. Louis, MO). Anti-Flag M2 beads (Sigma, St. Louis, MO) were added and lysates were incubated at 4°C overnight. The beads were washed six times with IP buffer and Flag Peptide (Sigma, St. Louis, MO) was added at 200 μ g/ml. Samples were eluted at room temperature and concentrated. Samples were then subjected to Western blotting analysis. Anti-DR5—TR2J (Human Genome Sciences) was crosslinked with anti-human IgG Fc (Sigma, St. Louis, MO) in a 1:1 ratio for 30 minutes prior to incubation with cells. Cells (2.5×10^7) were suspended in 25 ml of culture medium, incubated with TR2J/IgG at 1 μ g/ml at 4°C for 30 min, transferred to 37°C for another 1 hr, washed in phosphate-buffered saline three times, and then lysed in IP buffer for 1 hr at 4°C. After the lysates were centrifuged, lysates were precipitated at 4°C overnight. The beads were washed six times with IP buffer supplemented with 0.5 M NaCl and samples were subjected to Western blotting analysis.

Cell death assays

BJAB cells were plated in 96 well plates at 40,000 cells per well. TR2J (anti-DR5) and Mapatumumab (anti-DR4) both provided by Human Genome Sciences were cross-linked with anti-human IgG Fc for 30 min prior to serial dilution. The following drugs were prepared according to manufacturer's instructions and were applied in serial dilution format: TRAIL (R&D Systems, Minneapolis, MN), SuperFas Ligand (Enzo Life Sciences, Plymouth Meeting, PA), 5-Fluorouracil (5-FU), Doxorubicin Hydrochloride, Etoposide, Oxamflatin, Temozolomide, Sorbitol, MS-275, and Staurosporine (Sigma, St. Louis, MO), MG132 (EMD Biosciences, Gibbstown, NJ). UV irradiation was performed in a UV Stratalinker (Stratagene, La Jolla, CA) in a 24 well plate and then media and cells were transferred to a 96 well plate for MTS analysis after 48 hrs. An MTS Assay was performed after 24 hours incubation according to the manufacturer's (Promega, Madison, WI) recommendations. For long-term assays of cell survival/growth, 1 million cells expressing GFP control or FADD-DD were treated with TRAIL or etoposide for 24 hours, then washed and replaced into growth media for 7 days. Cell growth was

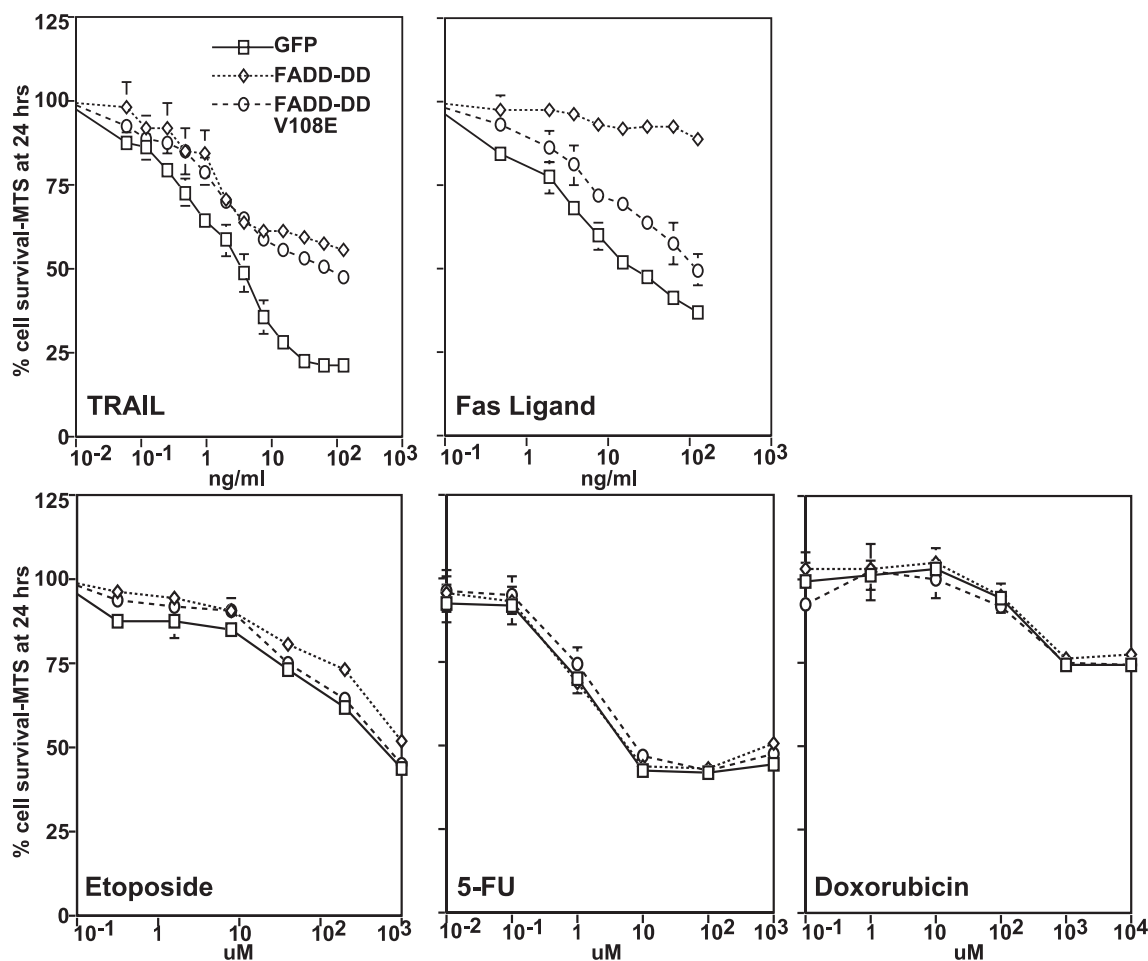


Figure 4. FADD-DD and FADD-DD V108E do not inhibit chemotherapy-induced death in HCT-116 cells. HCT-116 cells transfected with GFP, FADD-DD or FADD-DDV108E expression constructs were treated with increasing doses of TRAIL, FasL, etoposide, 5-FU, or doxorubicin as indicated and cell viability was assessed. FADD-DD and FADD-DDV108E inhibited FasL and TRAIL as in BJAB cells, but had no effect on tumor cell killing by the chemotherapy drugs.
doi:10.1371/journal.pone.0014527.g004

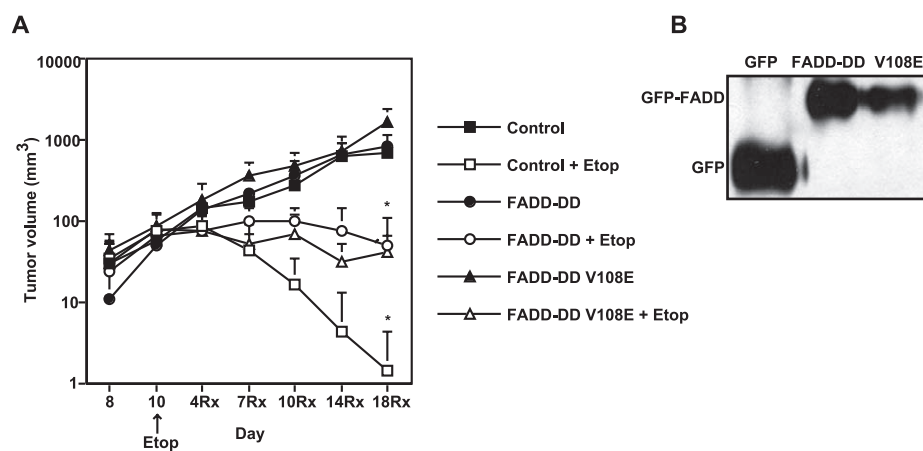


Figure 5. FADD-DD and FADD-DDV108E reduce the effectiveness of tumor eradication by etoposide in vivo. Panel A, isogenic BJAB cells expressing GFP control, GFP-FADD-DD and GFP-FADD-DDV108E were implanted subcutaneously and tumors grown for 10 days prior to treatment with etoposide. Untreated tumors continued to grow. In control BJAB cells, etoposide caused tumor eradication; whereas, in tumors expressing either FADD-DD or FADD-DDV108E, etoposide treatment led to stabilization of tumor mass but no eradication ($p < 0.05$ by t-test at 18 for the control versus FADD-DD and FADD-DDV108E expressing cells). Panel B, Western blot of tumor tissue from GFP control, GFP-FADD-DD and GFP-FADD-DD V108E demonstrating similar expression of the GFP-tagged protein in all tumors.
doi:10.1371/journal.pone.0014527.g005

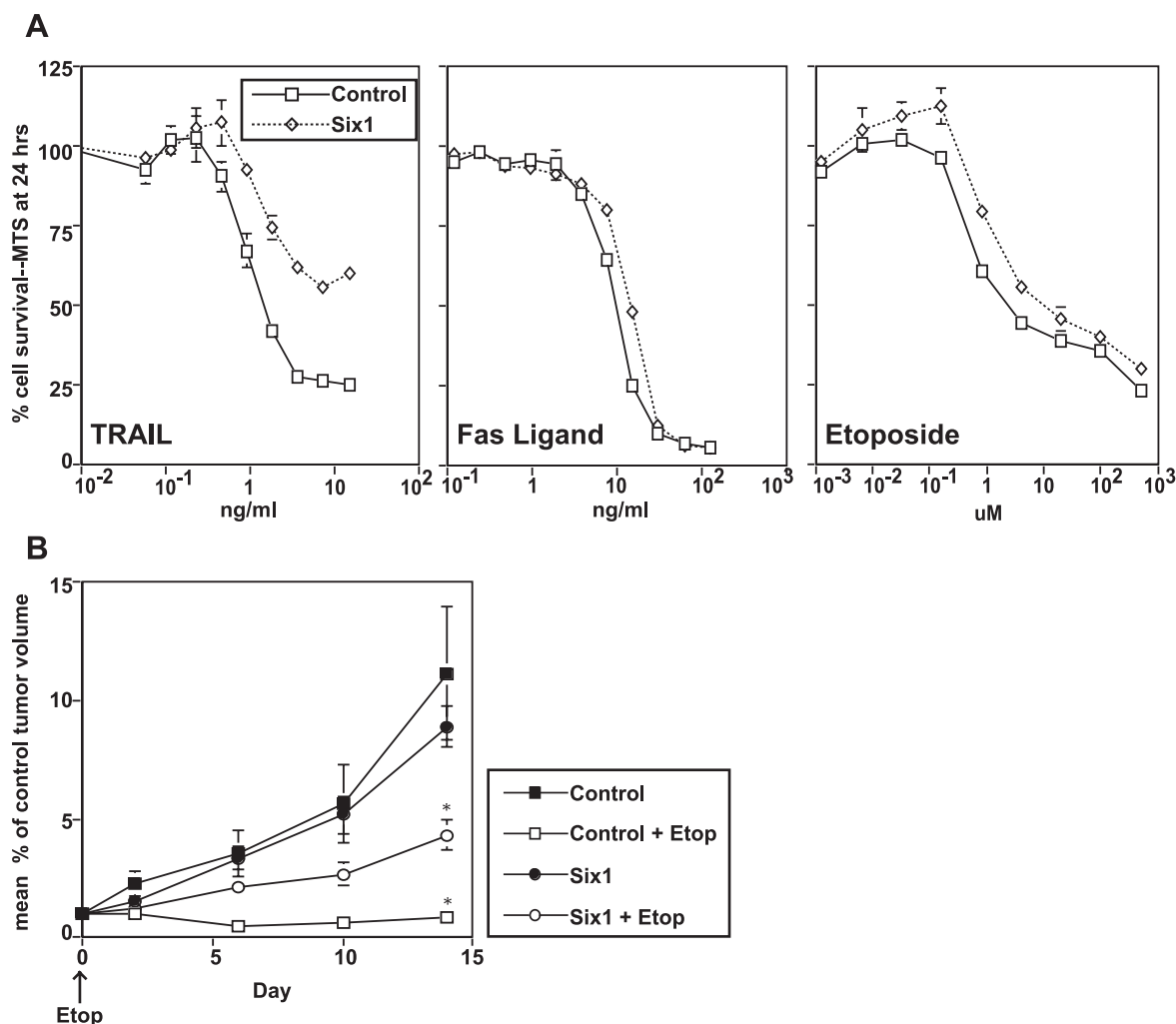


Figure 6. Six1-mediated TRAIL resistance reduces the effectiveness of etoposide in vivo, but not in vitro. Panel A, parental BJAB cells and BJAB cells expressing Six1 were treated in vitro with increasing doses of TRAIL, FasL and etoposide, and cell viability was assessed by the MTT assay. Six1 caused TRAIL resistance but had little effect on FasL or etoposide-induced cell death. Panel B illustrates how etoposide treatment of subcutaneous tumors from BJAB or BJAB-Six1 cells reduces the growth of both tumors relative to untreated controls, but is less effective in the Six1-expressing cells (* $p < 0.001$ by t-test at day 14 for the control versus Six1 expressing cells). doi:10.1371/journal.pone.0014527.g006

determined by counting viable cells; the ability of surviving cells to grow was demonstrated by an increase in the total number of cells.

Immunoblotting

Cells (1×10^6) were harvested and lysates were prepared by boiling in SDS buffer 5 min prior to gel electrophoresis. Lysates were resolved on 12% SDS-polyacrylamide gels. Proteins were transferred to Immobilon-P Transfer Membrane (Millipore Corporation, Bedford, MA). Blots were blocked with 5% milk in TBST and incubated with antibodies that recognize IKK α , phospho-JNK, JNK, Caspase 8, Caspase 3 (Cell Signaling Technologies, Danvers, MA), FADD (BD Biosciences, Franklin Lakes, NJ). Blots were then incubated with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technologies, Danvers, MA). Detection was performed using chemiluminescent ECL reagent (Millipore Corporation, Bedford, MA) and developed on Blue X-Ray film (Life Science Products, Inc., Frederick, CO).

Transfection of HCT116

Cells (1×10^6) were plated in a 6 well dish and transfected with GFP, GFP-FADD-DD, or GFP-FADD-DD V108E using Lipofectamine 2000 (Life Technologies Corporation, Carlsbad, CA). Transfection efficiency was verified using fluorescent microscope 24 hrs after transfection. Cells were trypsinized and replated at 16,000 cells per well in a 96 well plate and then allowed to sit down overnight. Cell death assays were then conducted.

Tumor Treatment studies

Groups of 3–4 NOD/SCID mice were subcutaneously injected at two sites/mouse with 1×10^7 BJAB cells and tumors allowed to grow to a size of $\sim 200 \text{ mm}^3$ prior to randomization into control or treatment (IP injection of etoposide (15 mg/kg twice a week) groups. Tumor size was monitored every other day using vernier digital calipers in three dimensions and calculated as a spheroid tumor volume ($h \times w \times l \times 0.523$). Tumor growth in the treated animals was compared between groups using t-test. For tumor western blotting, Paraffin-embedded tumors were deparaffinized in xylene, rehy-

drated in graded ethanol, immersed in distilled water, and air-dried. Tumors were diced into small pieces and homogenized in RIPA buffer containing 2% SDS. Samples were heated at 100°C for 20 min and then incubated at 60°C for 2 hrs. Debris was centrifuged twice to leave the supernatant for western blotting.

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Author Contributions

Conceived and designed the experiments: TG HLF KB AT. Performed the experiments: CM TG LQ CK. Analyzed the data: CM HLF KB AT. Contributed reagents/materials/analysis tools: CK. Wrote the paper: HLF KB AT.

Development and Prognostic Validation of a Polyclonal Antibody to Detect Six1 Homeoprotein in Ovarian Cancer

Lubna Qamar, Erin Deitsch, Aaron Patrick, Miriam D. Post, Andrew Thorburn, Heide L. Ford and Kian Behbakht

ABSTRACT:

Background: The Six1 homeobox gene encodes a homeodomain-containing transcription factor whose mRNA is a poor prognosticator in various malignancies including ovarian cancers. A specific Six1 antibody test has not been available to determine whether Six1 protein is a poor prognostic indicator, hence we describe validation of a Six1 specific antibody and evaluate its association with tumorigenicity and prognosis in ovarian cancer.

Methods: A specific Six1 antibody (Six1cTerm) was raised to residues downstream of the Six1 homeodomain, representing its unique C-terminus as compared to other Six family members. Cells were transfected with Six1-Six6 and western blot analysis was performed to demonstrate Six1 specificity. Ovarian cancer cell lines were analyzed for Six1 mRNA and Six1cTerm and tumorigenicity was evaluated in SCID mice. The metastatic tumors of 15 stage IIIC high grade serous ovarian cancers were analyzed and compared to clinical factors via t-test, Mann-Whitney U test, χ^2 test, and survival via the Kaplan-Meier method/Log Rank test.

Results: The Six1cTerm antibody is specific for Six1. Cell line tumorigenicity in SCID mice correlates with Six1 levels both by mRNA ($p=0.001$) and by protein (presence vs. absence, $p=0.03$). Six1 protein expression correlated with worsened survival ($p=0.003$).

Conclusions: The Six1cTerm antibody is specific and able to detect Six1 in cell lines and tumor tissue. Six1 protein detection is associated with tumorigenicity and poor prognosis in this cohort. Six1cTerm antibody should be further validated as a tool to convey prognostic information in a larger series of serous ovarian cancers.

Impact: This study demonstrates the poor prognostic impact of Six1 protein expression in ovarian cancer using a Six1 specific antibody.

INTRODUCTION:

Study of the expression of homeobox genes and their protein products has gained increasing importance in understanding the pathogenesis of human malignancies. Homeodomain-containing proteins act as transcription factors that regulate the coordinated expression of a variety of genes involved in development and differentiation(1-10). Interestingly, these genes are frequently inappropriately expressed in cancer(9-11). Six homeobox1(*Sine Oculis (so) homolog1*), also called Six1, belongs to a subfamily of the Six class of homeodomain-containing transcription factors and is an important developmental regulator that is necessary for the proliferation of precursor cell populations during formation of the muscle, kidney, and inner ear, among other organs(12-14). Six family genes are thought to have arisen from the multiplication of an ancestral Six gene that occurred prior to the evolution of the Bilateria(15). The three *Drosophila* Six genes *so*, *optix* and *Dsix4* have distinct functions, and these genes are further duplicated in the vertebrate lineage, resulting in the orthologs Six1/Six2(*so*), Six3/Six6 (*optix*) and Six4/Six5 (*DSix4*). The entire Six family of proteins share a highly conserved co-factor interacting Six domain (SD) and a highly conserved DNA binding Six-type homeodomain (HD), but otherwise have unique sequences and a non-conserved C-terminus(16). Within Six family subclasses, the C-terminus may be important in that it can confer functional specificity(17) .

Overexpression of Six1 is observed in several human cancers including breast, ovarian, uterine cervical, rhabdomyosarcoma, and hepatocellular carcinoma(9, 18-21). When Six1 is expressed outside of normal development, it appears to impart developmental properties on adult cells causing an increase in proliferation and metastasis and a decrease in basal and TRAIL-mediated apoptosis(10, 12, 18-19, 22). In ovarian cancer, Six1 mRNA is over-expressed in metastatic cancers as opposed to early stage cancers and postmenopausal normal ovaries and confers a poor prognosis independent of stage(18). However, mRNA is not very stable and its use as a prognostic marker requires fresh tissue. Analysis of Six1 homeoprotein expression would be preferable since protein is stable and can be easily analyzed in archival tissue, but has been hampered by the lack of a Six1 specific antibody. Due to the significant sequence homology in Six family members, antibodies that are raised to antigens containing the SD and/or HD regions are likely to cross-identify other family members, particularly within the same subclass(23). In this study, we report the development of a Six1 specific antibody raised to the unique C-terminal region of Six1. We show that this antibody can be used for immunohistochemistry and demonstrate its prognostic utility in that detection of Six1 protein correlates with ovarian cancer cell line tumorigenicity and with poor prognosis in advanced stage high grade serous ovarian cancers.

PATIENTS AND METHODS:

Antibody Production

The C-term anti-Six1 antibody was generated as previously described (Ford *et al.*, 2000). 2 mg of protein was sent to Proteintech (Chicago, IL) for antibody production in rabbits. The antibody was purified as described previously(24).

Cell Culture and Transfections

For analysis of the specificity of Six1 cTerm antibody, MCF7 cells were cultured as described(24) and then transfected with 10 µg each of human Six1 and Six2 (cloned into pcDNA3.1+, Invitrogen), and all mouse Six family members (a generous gift from Kiyoshi Kawakami) using Fugene 6 Transfection Reagent (Roche), according to the manufacturer's protocols. For analysis of Six1 staining, cell lines were grown on single chambered slides to 80% confluency in RPMI with 10% FBS. Cells were then fixed in methanol for 1hr at -20°C, rinsed with room temperature PBS and stained as described below. Cell lines for the analysis of Six1 mRNA and protein were grown on single chambered slides in RPMI media with 10% FBS and harvested in log phase growth.

Western Blot Analysis

48 h post-transfection, nuclear extracts were collected from the transfected cells according to Jamieson et al(25). Bradford assays were performed to determine the protein concentration, and 15 µg of protein from each extract was run on a 10% SDS-polyacrylamide gel. Western blotting was performed as described(24) using the following primary antibodies: 1:1000 C-term anti-Six1 (antibody described in this manuscript), 1:250 anti-Six1 (Sigma), 1:200 anti-Six4 (Abnova), 1:500 anti-Six5 (Bioworld), and 1:200 anti-Six6 (Affinity BioReagents). Secondary antibodies were 1:10,000 anti-rabbit or anti-mouse IgG horseradish peroxidase (Sigma), as appropriate. Chemiluminescence with SuperSignal West Pico (Thermo Scientific) was used to detect signal.

Immunohistochemistry

Human tissues were fixed in 10% neutral buffered formalin for 24 hours then embedded in paraffin. Sections were cut at 4µm and heat-immobilized at 60 degrees Celsius for 60 minutes on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). After deparaffinization, antigens were retrieved using Citra Plus 10X Retrieval Solution (BioGenex, San Ramon, CA) with a Biocare Medical Decloaker (Concord, CA) followed by 3% hydrogen peroxide for 5 minutes. After rinsing in PBS-T(0.1%), sections were blocked with 10% normal goat sera in TBS-T (0.1%), and were incubated with primary rabbit anti-hsix1 (1:1000) for 1 hour at room temperature. Rabbit IgG (invitrogen) was used as an isotype-negative control. Following three washes in TBS-T (0.1%), sections were incubated with biotinylated goat anti-rabbit IgG (1:1,000; DakoCytomation, Carpinteria, CA) for 30 minutes. Following washing, sections were incubated with streptavidin-conjugated horseradish peroxidase (1:1,000; DakoCytomation) for 30 minutes, washed, and then incubated with 3,3'-diaminobenzidine (DakoCytomation) for 10 minutes, rinsed in water, counterstained with dilute hematoxylin, dehydrated, and mounted for bright-field microscopy. Immunohistochemistry was done as described above with the following exceptions: slides were stained using Autostainer Universal Staining System (DakoCytomation) along with the Vectastain elite ABC kit (Vector Laboratories, Burlingame, CA). Stain intensity was scored on a scale of 0-3 (0-negative, 1-slight, 2-moderate, 3-intense) and a composite score was calculated by multiplying percent stained nuclei by the intensity score (range 0-300). Two blinded observers (K.B.

and M.P.) scored the slides independently and inter-observer reliability (κ statistic) was calculated.

Quantitative real-time RT-PCR.

Total RNA was extracted from patient specimens stored in RNAlater using standard TRIzol extraction (Invitrogen/Life Technologies, Carlsbad, CA). Purity, concentration, and integrity of total RNA was verified using a spectrophotometer as well as the RNA 6000 Nano assay (Agilent Technologies, Palo Alto, CA) and visualization of the 18S and 28S rRNA bands. For quantitative RT-PCR, 1 μ g of extracted RNA was analyzed by rRNA amplification to verify integrity of the RNA. High-quality specimens were then analyzed for Six1 mRNA levels using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) and Six1-specific primers and Taqman probes. Results are measured as fg Six1/ng 18S rRNA sample divided by fg/ six1 /ng 18S rRNA of log phase growing SKOV3 cells and reported as percentage of (SKOV3) reference from the same experiment (Relative to SKOV3 Reference, RSR).

Tumorigenicity Analysis

10 million log phase growing cells were injected into each flank of triplicate CB-17 SCID mice (taconic) using an IACUC approved protocol. Mice were evaluated daily and three dimensional tumor measurements were obtained three times weekly using calipers. Each experiment was repeated at least once. Mice were euthanized if they became moribund or lost greater than 15% of starting weight.

Patient Tumor Data collection

Between January 2003 and June 2008, fresh omental or peritoneal metastases of 15 patients with stage IIIC high grade serous ovarian cancer were obtained under an IRB approved protocol and high-quality RNA was extracted as above. All patients underwent an exploratory laparotomy with the intent of performing total abdominal hysterectomy (when the uterus was present), bilateral salpingo-oophorectomy, omentectomy, and aggressive tumor cytoreduction. All patients were treated with post-operative platinum-based chemotherapy. All pathology was reviewed by the University of Colorado Anschutz Medical Campus gynecologic pathologists and reviewed again by a single pathologist at the institution's weekly Gynecologic Oncology Tumor Board. Archival tumors from the same specimen as the extracted RNA above were obtained subsequently and evaluated by H & E and immunohistochemistry on 5-8 μ m sections as above. Clinical variables abstracted included age, stage of disease, tumor histology, pre-operative CA125 levels, extent of residual disease and tumor response to platinum chemotherapy as measured by a recurrence free interval of greater than 6 months. Variables were compared with the t-test, Mann-Whitney U test and χ^2 and Fischer's Exact test. Survival was analyzed by the Kaplan-Meier Method with comparisons via the Log Rank test.

RESULTS

Because of the high degree of conservation between Six family members, we first verified the specificity of the Six1cTerm antibody for Six1 using Western blot analysis and after transfecting each Six family member into MCF7 cells. Figure 1 (cropped) shows that the Six1cTerm antibody is specific for human Six1 while not cross-reacting with the most conserved (as compared to Six1) Six2 protein or any other mouse Six family members. Human Six3-Six6 do not contain the C-terminus used to generate this antibody and were not analyzed. Supplemental Figure 1 shows the full blots from which Figure 1 was generated. Additionally in Supplemental Figure 1C, a commercially available Six1 antibody, the Sigma anti-Six1 antibody, is shown to cross react with human and mouse Six2 as well as mouse Six3.

To determine whether the Six1 specific antibody could detect Six1 using immunocytochemical methods, we utilized our previously generated Caov3/Six1 overexpressing clones and our Caov3/CAT control clones(18), which express high and low levels of Six1 respectively. Figure 2 demonstrates that Six1cTerm antibody detects increased levels of exogenous Six1 protein, in corroboration with the level of mRNA detected.

To assess the ability of the Six1cTerm antibody to detect endogenous Six1, a panel of ovarian cancer cells lines available from the gynecologic tumor bank at the University of Colorado and with varied levels of Six1 mRNA (Table 1) were selected for Six1cTerm staining. Additionally, given previous data showing that expression of Six1 in non-tumorigenic MCF12A breast cancer cells can induce tumor formation(26), the panel of cell lines were assayed for tumorigenicity in SCID mice to correlate tumorigenic capability with Six1 expression (Table 1). Median cell line Six1 expression was 0.39 RSR (Relative to SKOV3 Reference) with a range of 0-10.0 RSR. All cell lines with $\text{Six1} \geq 0.37$ RSR were tumorigenic while all with Six1 expression below 0.37 RSR were not tumorigenic. Hence, Six1 mRNA expression was significantly correlated with SCID mouse tumorigenicity ($p= 0.001$ Mann-Whitney U test). Six1cTerm detection (any nuclear staining, composite score > 0 with agreement by both evaluators) also correlated with tumorigenicity ($p= 0.03$ Fischer's Exact test). The A2780 cell line displayed intense staining with composite score of 300 and Six1 mRNA RSR of 10.0 ± 3.0 . The next highest Six1 mRNA RSR level of 1.0 (SKOV3) did not express consistent nuclear staining as compared to negative control and the remainder of the Six1cTerm positive cell lines had slight staining. Correlation of composite score to mRNA was not possible. Representative photographs of Six1 staining for the OV429 (Six1cTerm negative), DOV13 (Six1cTerm negative), CaOV3 (Six1cTerm positive), OVCAR2(Six1cTerm positive), SKOV3 (Six1cTerm negative) and A2780 (cTerm positive) cell lines are shown in figure 3.

To explore whether Six1 protein levels could be useful as a prognostic indicator, archived tumor blocks of 15 patients with stage IIIC high grade serous ovarian cancer were obtained under an IRB approved protocol, stained for Six1, and compared to qRT-PCR results available from the same patients. The mean age of patients enrolled was 60

(range, 48-76). All patients had high grade serous stage IIIC ovarian cancer and elevated CA125 > 20 mIU/ml (mean 831 mIU/ml, range 25-3907 mIU/ml). Mean tumor Six1 mRNA from omental or peritoneal metastases was 0.69 (range 0-4.2 relative to reference) and 3/15 specimens had detectable Six1 protein by Six1cTerm (score > 0 with agreement by both slide reviewers). Interobserver correlation between the two blinded slide reviewers (K.B., M.D.P.) was substantial ($\kappa = 0.76$). Age, preoperative CA125, extent of residual disease and tumor response to platinum chemotherapy was not different between patients whose tumors expressed Six1 protein versus those whose tumors did not express Six1 protein. Figure 4 shows Kaplan-Meier survival curves by Six1 protein and mRNA level. At 34 months of median follow-up, median survival for the patients whose tumors did not have Six1cTerm staining had not been reached as compared to 24 months (95% C.I. 12-37 months) for those whose tumors were positive for Six1cTerm ($p = 0.003$ log rank test). Mean survivals were 63 months (95% C.I. 57-70 months) for those without staining as opposed to 24 months (95% C.I. 17-33 months) for those whose tumors stained positive for Six1. mRNA data from the same patient data set revealed a similar but non-significant trend (Figure 4B).

DISCUSSION:

We have developed a Six1 specific C-terminal antibody that can detect Six1 reliably and specifically in archived tissue samples and whose expression can yield prognostic information. Our findings are significant in that prior publications using anti-Six1 antibodies for immunohistochemistry have described the possibility of cross-reactivity to other Six family members when antibodies used were raised to fragments containing the Six SD or HD regions(23). Expression of other Six family members can be mistaken for Six1 expression, confounding results. For example, Six6 is overexpressed in acute T-Cell leukemias(27) while Six3 is downregulated in gastric cancer, yet overexpressed in extraskeletal myxoid chondrosarcoma (28-29). Additionally, Six5 is overexpressed in low malignant potential ovarian tumors(30). As a subset of invasive low grade ovarian tumors share genetic aberrations with their low malignant potential precursors, it is possible that a non-specific antibody may erroneously identify Six1 expression when one of the other Six family members, such as Six5, is elevated. Furthermore, as seen in the mammary glands of Six1-/Six1- knockout mice, inducing loss of Six1 is associated with increase in other family members such as Six2 and Six4(31). A non-specific Six1 protein marker would not be able to discern the successful knockdown of Six1 in this scenario, picking up Six2 or Six4 staining in error.

Six1 expression is correlated with increased malignancy and worse prognosis in cancers as diverse as B cell lymphoma and oligodendroglioma(32). However, mRNA overexpression is commonly less than 3 fold(23). In a larger sample of patients with ovarian cancer, Six1 mRNA was over-expressed in metastatic cancers compared to early stage cancers and postmenopausal normal ovaries and conferred a poor prognosis independent of stage(18). However, the use of mRNA as a prognostic marker is clinically limited by the need for fresh tissue for analysis, the inability to accurately separate tumor and normal tissue, and by the need for an extremely sensitive PCR test

that can accurately detect small changes in mRNA expression. Thus, a Six1 specific antibody that can be utilized for immunohistochemical analysis of paraffin embedded samples allows the use of readily available archival tissue and facilitates visualization of tumor versus normal tissue staining. While in a larger sample of tumors from ovarian cancer patients, we have previously shown that Six1 mRNA is prognostically important, here we shown prognostic importance of Six1cTerm in a smaller patient sample. While our study was not designed for a direct prognostic comparison of Six1 mRNA versus protein, it does suggest that protein analysis by Six1cTerm is possible and may be prognostic.

We have characterized the Six1 cTerm antibody and have demonstrated its Six1 specificity. The antibody was generated against the c-terminus of Six1 in an attempt to generate a Six1 specific antibody by excluding the highly similar SD and HD regions shared by the other Six family members. This was especially critical for generating an antibody that would not cross-react with Six1's subfamily member Six2, which shares 93% and 98% amino acid identity with Six1 in the SD and HD respectively. Our Six1 cTerm antibody was able to detect human and mouse Six1 by Western Blot analysis while not cross reacting with human or mouse Six2.

The Six1cTerm antibody was able to generate a strong and specific reaction that was clearly associated with Six1 mRNA levels in ovarian cancer cell lines overexpressing Six1. Displaying an expected Six1 phenotype, both Six1 mRNA and Six1cTerm staining were associated with tumorigenicity in ovarian cancer cell lines. Finally, we demonstrated the utility of Six1cTerm antibody by the Six1 staining of a panel of 14 ovarian cancer cell lines with varied levels of Six1 mRNA. In further development, the Six1cTerm antibody could be used to give pre-operative or pre-chemotherapy prognostic information via a small volume biopsy or assessment of ascites commonly seen with ovarian cancer. Additionally, given the small cell number requirements of immunohistochemistry as compared to mRNA analysis, circulating tumor cells, which are known to be present in the blood of ovarian cancer patients(33), can be queried with Six1cTerm antibody to yield prognostic information.

We have previously shown poor survival with Six1 expression in a series of advanced ovarian cancer patients with varied histologic subtypes(18). Given information about significant variances in ovarian cancer molecular subtypes(34), we have selected the most common and the most deadly variety, high grade serous ovarian cancer, in the most common substage, IIIC, for this analysis. Here, we show that Six1 protein expression is associated with decreased survival, even in as few as a 15 patient cohort. The Six1 cTerm polyclonal antibody could therefore be a useful tool as a prognostic indicator in high grade serous ovarian cancers and should be validated in a larger sample of ovarian cancers.

FIGURE LEGENDS:

Figure 1. Western blot analysis on nuclear extracts from MCF7 cells transfected with mouse (A) and human (B) Six family members. For the analysis, an anti-Six1 antibody developed against the C-terminus of the protein was used. Images have been cropped for inclusion in this figure. Full-length blots, as well as verification of the presence of all Six family members on the membranes (using specific Six family antibodies and antibodies that cross-react with several Six family members), are included in supplemental figure 1. Bottom panel demonstrates probing of the same membrane with an HDAC antibody as a loading control.

Figure 2. Six1 immunohistochemistry with the cTerm antibody at 1:500 of the CaOV3-CAT clones (panels A and B) and CaOV3-Six1 clones (panels C and D). Insets for each panel are negative controls.

Figure 3. Six1 immunohistochemistry with the cTerm antibody at 1:500 OV429 (Six1 mRNA = 0 RSR, panel A), DOV13 (Six1 mRNA 0.25 RSR, Panel B), CaOV3 (Six1 mRNA = 0.42 RSR, panel C), OVCAR2 (Six1 mRNA 0.6 RSR, Panel D), SKOV3 (Six1 mRNA = 1.0 RSR, panel E) and A2780 cell lines (Six1 mRNA = 10.0 RSR, panel F). Insets for each panel are negative controls.

Figure 4. A. Six1 protein expression and the survival of patients with high grade serous Stage IIIC Ovarian Cancer. The survival difference is statistically significant (Log Rank $p=0.003$). B. Six1 mRNA expression (1 equals >0.75 RSR, 0 equals ≤ 0.75 RSR) from the same patients predicts a survival trend, but is less powerful than protein analysis.

Supplemental Figure 1. Western blot analysis on nuclear extracts from MCF7 cells transfected with mouse (A) and human (B) Six family members. For the analysis, an anti-Six1 antibody developed against the C-terminus of the protein was used, as well as an anti-HDAC antibody (panels A and B). Blots were also probed with specific and/or cross-reactive antibodies for each Six family member to show presence of each family member in extracts from transfected cells. (C)

Figure 1.

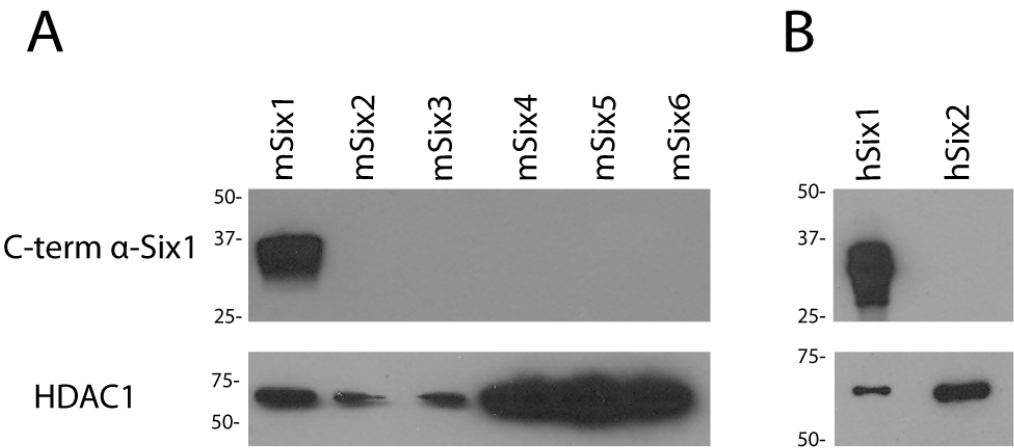


Table 1. Six1 mRNA expression and SCID mouse tumorigenicity in a panel of ovarian cancer cell lines with a range of mRNA expression

Cell Line	Description/Reference	Six1 mRNA (% of SKOV3 reference)	cTerm protein	Tumors in SCID mice
OV429	Serous Ovarian Cancer(35)	0.005 ± 0.004	Negative	No
OV420	Serous Ovarian Cancer(35)	0.02 ± 0.02	Negative	No
OVCAR5	Ovarian Cancer Ascites(36)	0.07 ± 0.04	Negative	No
OV432	Serous Ovarian Cancer(35)	0.14 ± 0.13	Negative	No
2008	Ovarian Cancer(37)	0.20 ± 0.09	Negative	No
DOV-13	Ovarian Cancer(38)	$0.25 \pm .16$	Negative	No
OV1847	Ovarian Cancer	$0.37 \pm .17$	Negative	Yes
CaOV3	Ovarian Cancer(39)	$0.42 \pm .14$	Positive	Yes
OVCAR2	Ovarian Cancer Ascites(36)	$0.60 \pm .13$	Positive	Yes
PECOC167	Serous Ovarian Cancer	$0.63 \pm .06$	Negative	Yes
HeyC2	Serous Ovarian Cancer(40)	$0.81 \pm .06$	Positive	Yes
Hey	Serous Ovarian Cancer(39)	1.0 ± 0.16	Positive	Yes
SKOV3	Grade 2 Ovarian Cancer(39)	1.0 (reference)	Negative	Yes
A2780	Ovarian Cancer(41)	10.0 ± 3.0	Positive	Yes

Figure 2.

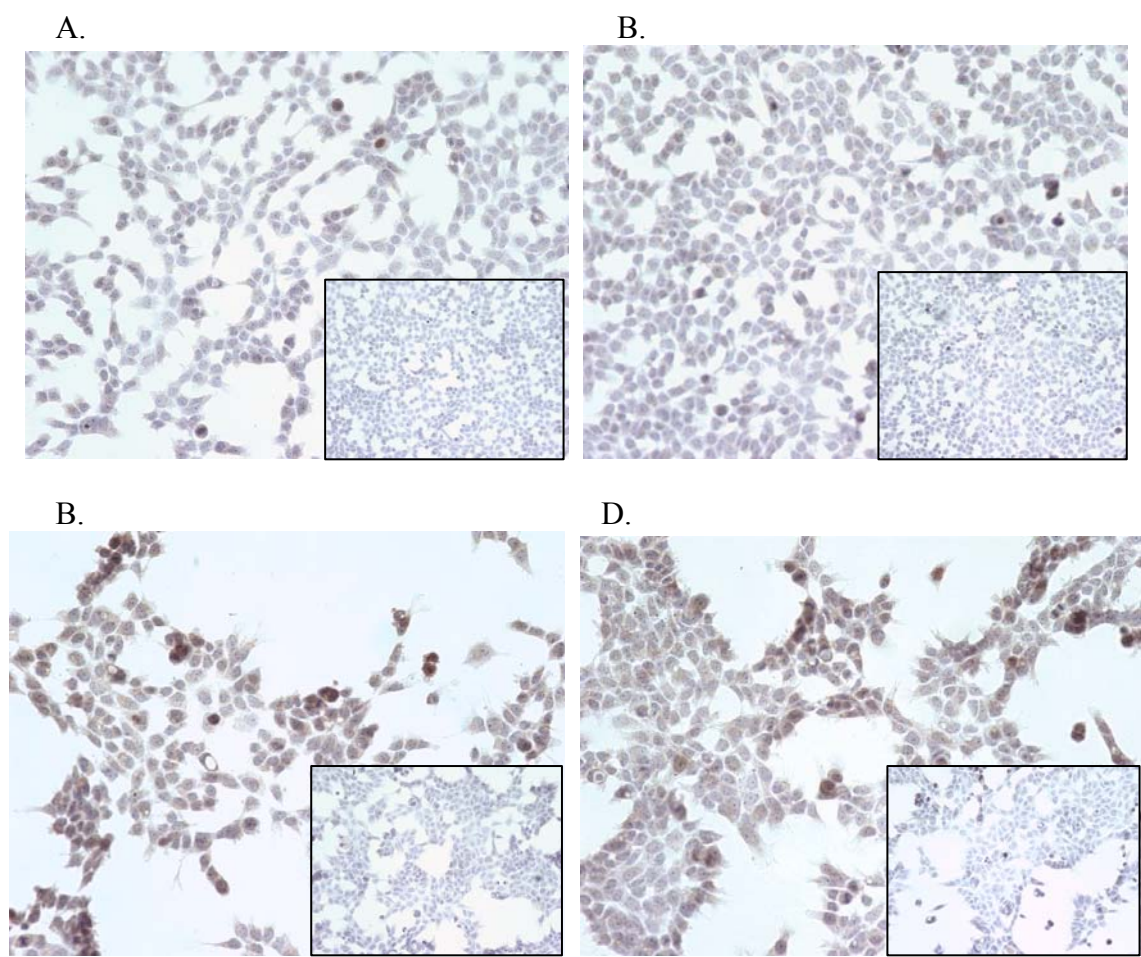
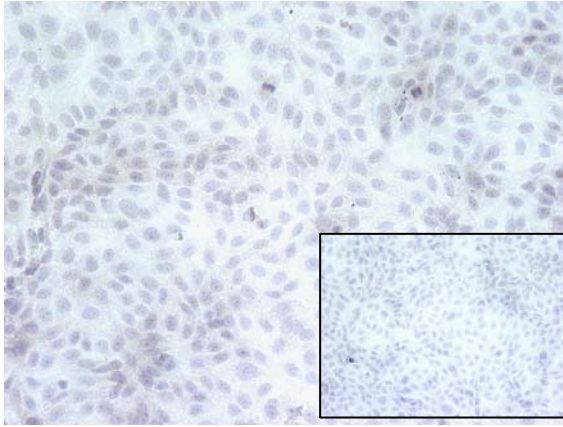
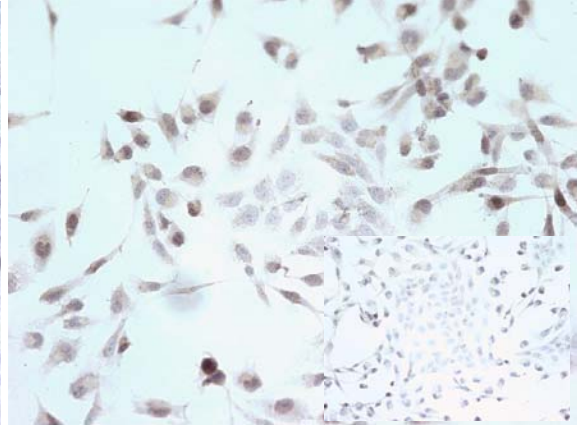


Figure 3.

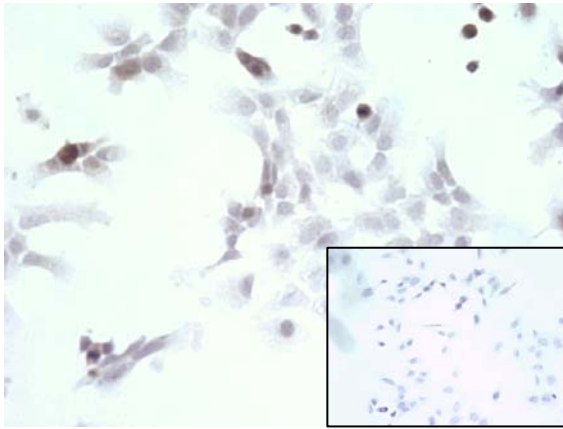




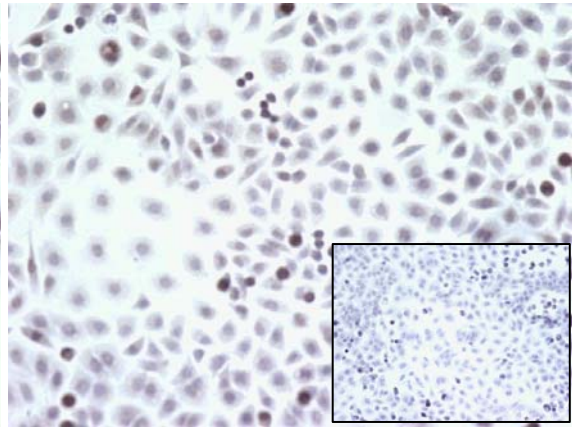
C.



D.



E.



F.

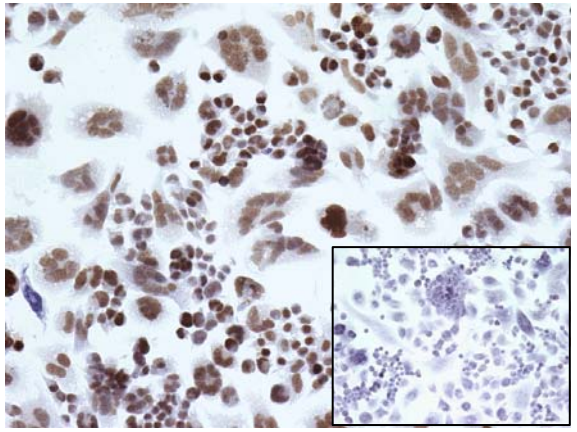
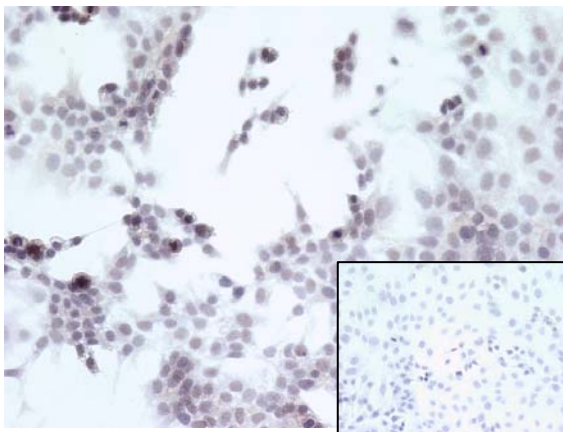
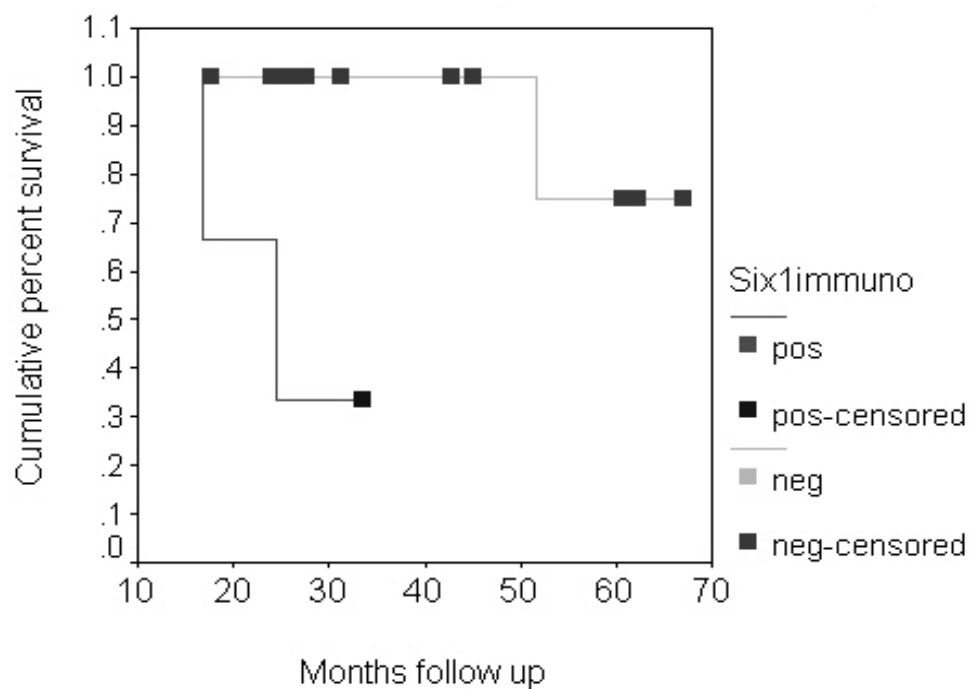
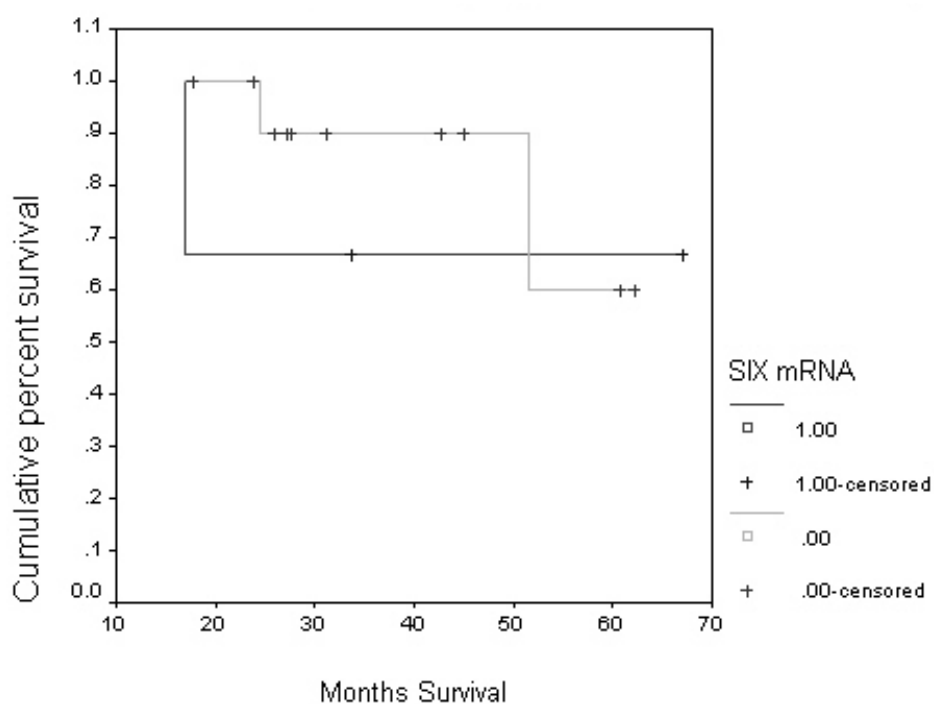


Figure 4.

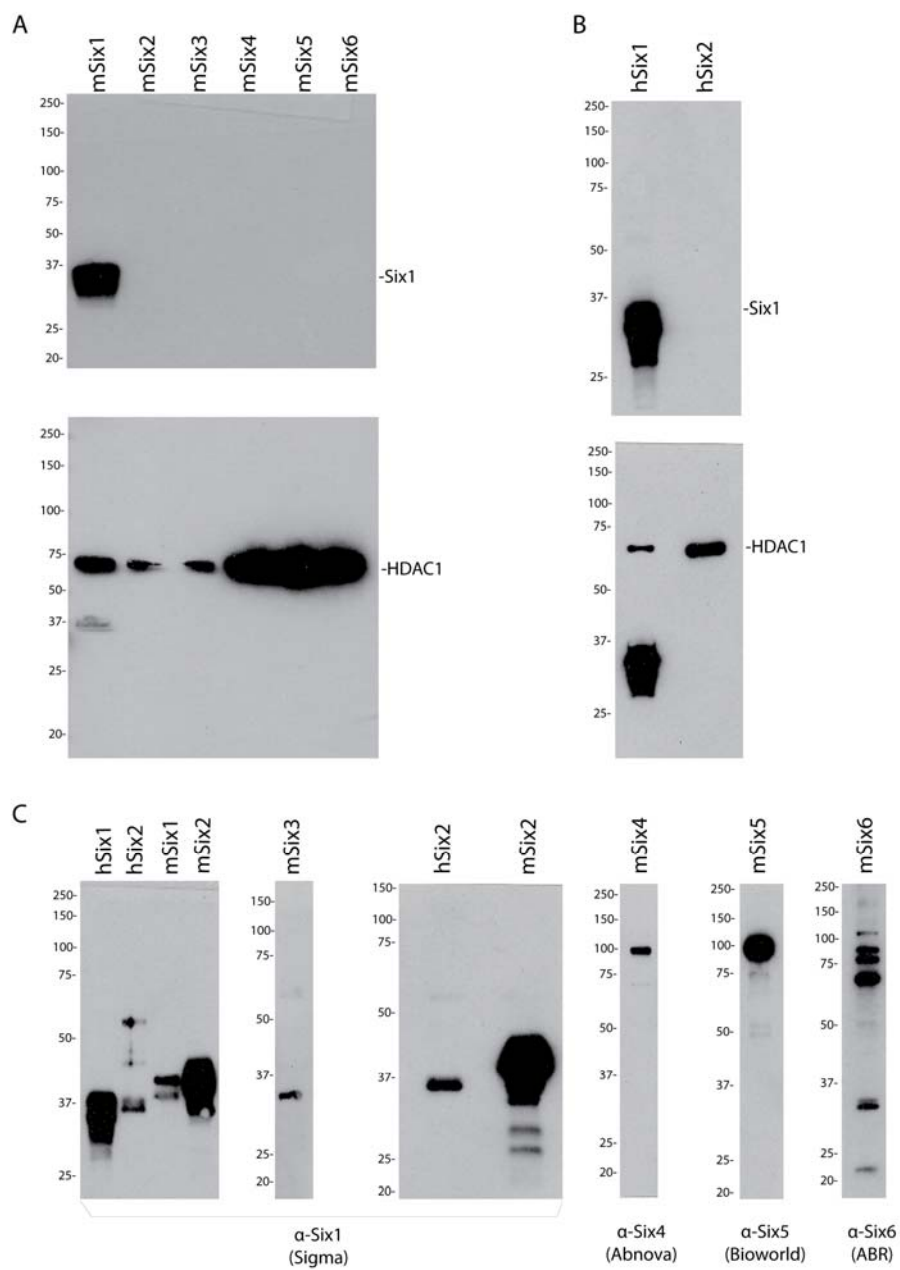
A.



B.



Supplemental Figure 1.



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Conclusions: Clinicians struggle in their efforts to distinguish patients with recurrent ovarian cancer who have potentially reversible and treatable problems from those who are entering a terminal phase of their illness. In the final 100 days of an ovarian cancer patient's life, the disease produces distinct symptoms requiring management and resource utilization. Our data suggest that even as disease progresses, we are inclined to perform evaluations and offer treatments, as well as offer care to provide symptom management. Worsening gastrointestinal symptoms or increased use of hospital admission or procedures should identify patients as potentially moving toward the final phases of their illness.

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The search for meaning, symptoms and transvaginal ultrasonography screening for ovarian cancer: Predicting malignancy

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Objectives: The mortality rate of ovarian cancer is greater than that of all other major gynecologic malignancies. Most women present with advanced-stage disease, where response to treatment is limited and prognosis is poor. Detecting ovarian cancer at an early stage, when it is curable, has long been an important goal of gynecologic oncologists. Recently, it has been reported that certain symptom patterns can be informative for the presence of ovarian malignancy. The present investigation was performed to determine how well symptoms and ultrasound findings would predict ovarian malignancy individually or in combination.

Methods: A group of 450 women, all of whom received surgery due to participation in annual transvaginal ultrasonography (TVS) screening, were selected from 31,748 women enrolled. Symptom questionnaires were provided, and the tabulated results were compared with ultrasound reports and surgical pathology for 272 of the women.

Results: Thirty malignancies and 420 persisting benign tumors constituted the group under study. The ability to distinguish malignant from benign ovarian tumors was based on sensitivities, specificities, and ROC curve analysis. TVS performed better than symptom analysis for detecting malignancies (73.3% vs 20% sensitivity), and symptom analysis performed better for distinguishing benign tumors (91.3% vs 74.4% specificity). Decisions based on simultaneously meeting TVS and symptom criteria resulted in poorer identification of malignancy in ROC analysis (with Morphology Index (MI) >5 and symptom analysis, sensitivity=16.7%), but improved the ability to distinguish benign tumors (with MI>5 and symptom analysis, specificity=97.9%). Decisions based on satisfying either symptom criteria or TVS criteria had small increases in sensitivity (+3.3%) and coordinated small decreases in specificity (-5.8%).

Conclusions: Symptom analysis does identify malignant ovarian tumors, but its discrimination by itself is inferior to that of TVS. The clinical significance of the findings reported here is that: (1) a screen that is negative by both ultrasound and the symptom index is likely to indicate a benign tumor (specificity >97%), and (2) adding symptom information with equal weight as ultrasound slightly improves the discrimination of malignancy (one additional TP with a sensitivity increase=+3.3%). These results strongly indicate that the major screening benefit in discriminating malignancy is achieved via ultrasound tools, whereas symptom information can aid in reducing surgery on women with benign conditions that generate ultrasound abnormality. Combining symptom analysis with TVS improved the discrimination of benign tumors, but it is coordinated with much poorer discrimination of malignant tumors, indicating that informative symptoms can be expected to be absent in a large fraction (80%) of ovarian malignancies.

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The six1 homeobox gene is associated with resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in ovarian cancers and is correlated with increased TRAIL decoy receptor DcR2 in a six1 overexpression model

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Objectives: Ovarian cancers express TRAIL receptors and TRAIL synergizes with chemotherapy in ovarian cancers. However, up to 60% of ovarian cancers overexpress the Six1 homeobox gene and we have shown that Six1-overexpressing ovarian cancers are resistant to TRAIL. To assess the role of TRAIL decoy receptors in Six1-related TRAIL resistance, we studied the expression of TRAIL and TRAIL decoy receptors and correlated these with Six1 expression and dose response to TRAIL and TRAIL receptor agonists.

Methods: Six1 expression and TRAIL receptor DR4 and DR5 and decoy receptor DcR1 and DcR2 mRNA levels were analyzed by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) in a panel of 15 ovarian cancer cell lines as well as Six1 stable transfected CaOV3 clones and control CAT clones. Dose-response curves were generated to TRAIL, FasL (as a control for non TRAIL receptor-induced apoptosis) and agonistic antibodies to TRAIL DR4 and DR5 and correlated with Six1 and TRAIL receptor expression.

Results: All 15 cell lines expressed DR4 (mean=123 ± 77 ag/ng rRNA), DR5 (mean=210 ± 106 ag/ng rRNA) and DcR2 (mean=81 ± 63 ag/ng rRNA), but only once cell line expressed DcR1. Six1 expression (overexpression vs underexpression, mean=108 fg/ng rRNA, range: 0-763) correlated with TRAIL resistance (TRAIL IC₅₀ > 100 ng/mL, $P=0.05$, χ^2 test) and all cell lines sensitive to TRAIL were also sensitive to anti-DR5

antibody ($IC_{50} < 1000$ ng/mL), but not sensitive to anti-DR4 antibody. Cells were resistant to FasL. Although Six1 mRNA compared across all cell lines did not correlate with DcR2 expression, stable Six1 overexpression in the low-Six1, low-DcR2-expressing CaOV3 cell line increased TRAIL IC_{50} fivefold and significantly increased DcR2, whereas DcR1 levels were unchanged.

Conclusions: Ovarian cancer cells express TRAIL DR4 and DR5 and the decoy receptor DcR2. Decoy receptor DcR1 expression is uncommon. Six1 expression correlates with DcR2 expression and TRAIL resistance in a CaOV3 Six1 overexpression model. The Six1-correlated increase in the TRAIL decoy receptor DcR2 may be a mechanism for TRAIL resistance in ovarian cancers. Given the relationship between Six1 expression and TRAIL resistance, the lack of a direct correlation between Six1 and DcR2 across all cell lines implies other Six1-driven TRAIL resistance mechanisms as well. Additional Six1 overexpression and knockdown experiments are underway.

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The utility of physical examination in detecting recurrence in patients with advanced epithelial ovarian cancer

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Objectives: The purpose of this study was to assess the value of physical examination in detecting recurrence in patients with advanced epithelial ovarian cancer in complete remission.

Methods: This was a retrospective study of patients with stage IIIC and IV epithelial ovarian cancer diagnosed between 1997 and 2005 who underwent primary surgical debulking followed by adjuvant chemotherapy. We included only patients who had a complete response to adjuvant chemotherapy with no evidence of disease on physical examination, CA-125 determination, and CT scan (when available) and who suffered from recurrence of their disease while under our care.

Results: Seventy-nine patients fit the inclusion criteria. Median age was 59.8 (range: 30–89). Seventy-one patients (89.9%) had stage IIIC and eight (10.1%) had stage IV. Seventy-four patients (93.7%) had papillary serous, two (2.6%) had clear cell, two (2.6%) had endometrioid, and one (1.3%) had mucinous adenocarcinoma. Seventy-seven patients (97.5%) had grade 3, one patient had grade 2, and one had grade 1. Preoperative CA-125 levels were available for 74 patients with a median of 537 (range: 17–25,224) U/mL; six of the 74 had normal preoperative levels (< 35 U/mL). The first evidence of recurrence was CA-125 elevation in 62 patients (78.5%), positive clinical findings on physical examination in 9 patients (11.4%), positive CT scan in seven patients (8.9%), and one patient was incidentally found to have recurrent carcinoma in the hernia sac during hernia repair. Of the 9 patients who were first diagnosed with recurrence based on positive clinical findings, seven (77.7%) had significant symptoms that prompted the physical examination (two had

bowel obstruction, two had neurologic symptoms, one had flank pain, one had a groin mass, one had a new large breast mass). Two patients had asymptomatic recurrences first found on physical examination during a routine follow-up visit; however, one had an elevated CA-125 and the other had an abnormal CT scan and both of these tests were already scheduled on the same day as the physical exam.

Conclusions: Physical examination has limited utility in detecting ovarian cancer recurrence during routine follow-up visits. Patients with an initial clinically diagnosed recurrence either were symptomatic or had concurrent positive routine CT scan or CA-125. Changing the routinely scheduled follow-up visits to an as-needed basis may be more convenient and economical in patients with epithelial ovarian cancer in remission.

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Therapeutic efficacy of folate receptor α blockade with MORAb-003 in ovarian cancer

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Objectives: The relative overexpression of folate receptor α (FR α) in ovarian cancer compared with normal tissues offers opportunities for novel therapeutic approaches to ovarian cancer. The purpose of this study was to examine the functional significance of FR α blockade with a novel monoclonal antibody, MORAb-003.

Methods: FR α expression was examined in ovarian cell lines (SKOV3ip1, IGROV, HeyA8, A2780-par, and HIO-180) with fluorescence-activated cell sorting analysis. In vitro (cell viability, migration, invasion) and in vivo (tumor growth) effects of FR α blockade on ovarian cancer cells were examined using well-characterized models. The mechanistic effects on the src-family nonreceptor tyrosine kinase Lyn were also examined.

Results: IGROV and SKOV3ip1 cell lines both expressed high levels of FR α compared with the non-transformed (HIO-180) cells. HeyA8 and A2780-par cell lines lacked FR α expression. In vivo, MORAb-003 led to 44 and 84% decreases in tumor growth in SKOV3ip1 and IGROV, respectively, when compared with control IgG antibody. Compared with other groups, the greatest efficacy was noted in the MORAb-003 plus docetaxel group (96 and 99% decreased tumor growth for SKOV3ip1 and IGROV compared with controls, $P < 0.001$). In the IGROV model, treatment with MORAb-003 resulted in a 27% decrease in tumor cell proliferation by PCNA staining ($P < 0.001$). MORAb-003 redistributed active, phosphorylated Lyn kinase out of lipid rafts, with a 60% decrease in active Lyn compared with control antibody. MORAb-003 did not significantly affect SKOV3ip1 cell viability, migration or invasion in vitro.

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TITLE: The Six1 Homeobox gene is associated with TRAIL resistance in ovarian cancers and is correlated with increased Tumor Necrosis Factor-Related Apoptosis Inducing Ligand (TRAIL) decoy receptor DcR2 in a Six1 over-expression model.

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ABSTRACT BODY:

Objectives : Ovarian cancers express TRAIL receptors and TRAIL synergizes with chemotherapy in ovarian cancers. However, up to 60% of ovarian cancers overexpress the Six1 homeobox gene and we have shown that Six1 overexpressing ovarian cancers are resistant to TRAIL. In order to assess the role of TRAIL decoy receptors in Six1-related TRAIL resistance, we studied the expression of TRAIL and TRAIL decoy receptors and correlated these with Six1 expression and dose response to TRAIL and TRAIL receptor agonists.

Methods : Six1 expression and TRAIL receptor DR4, DR5 and decoy receptor DcR1 and DcR2 mRNA levels were analyzed by real time quantitative RT-PCR (qRT-PCR) in a panel of 15 ovarian cancer cell lines as well as Six1 stable transfected CaOV3 clones and control CAT clones. Dose response curves were generated to TRAIL, FasL (as a control for non TRAIL receptor induced apoptosis) and agonistic antibodies to TRAIL DR4 and DR5 and correlated with Six1 and TRAIL receptor expression.

Results : All 15 cell lines expressed DR4 (mean 123 +/- 77 ag/ng rRNA), DR5 (mean 210 +/- 106 ag/ng rRNA) and DcR2 (mean 81 +/- 63 ag/ng rRNA) but only once cell line expressed DcR1. Six1 expression (over vs. under mean, mean 108 fg/ng rRNA, range 0-763 fg/ng rRNA) correlated with TRAIL resistance (TRAIL IC50 > 100 ng/ml, p= 0.05 Chi Squared) and all cell lines sensitive to TRAIL were also sensitive to anti-DR5 antibody (IC50 <1000 ng/ml) but not sensitive to anti-DR4 antibody. Cells were resistant to FasL. Although Six1 mRNA compared across all cell lines did not correlate with DcR2 expression, stable Six1 overexpression in the low Six1, low DcR2 expressing CaOV3 cell line increased TRAIL IC50 5 fold and significantly increased DcR2 while DcR1 levels were unchanged.

Conclusions : Ovarian cancer cells express TRAIL DR4 and DR5 and the decoy receptor DcR2. Decoy receptor DcR1 expression is uncommon. Six1 expression correlates with DcR2 expression and TRAIL resistance in a CaOV3 Six1 over-expression model. Six1 correlated increase in the TRAIL decoy receptor DcR2 may be a mechanism for TRAIL resistance in ovarian cancers. Given the relationship between Six1 expression and TRAIL resistance, the

lack of direct correlation between Six1 and DcR2 across all cell lines implies other Six1-driven TRAIL resistance mechanisms as well. Additional Six1 overexpression and knockdown experiments are underway.

TABLE TITLE: (No Tables)
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TITLE: Using a *Ex-vivo* Tumor Slice System to Investigate Experimental Therapies in Epithelial Ovarian Carcinoma

AUTHORS: Charles Anderson M.D., Lubna Qamar, Ph.D., Susan A Davidson, M.D., Monique Spillman, M.D. Ph.D., Kian Behbakht M.D.

OBJECTIVE(S): Preclinical investigation of experimental cancer therapies using cell culture or murine xenograft models are often limited by expense and poor correlation with eventual outcome. We sought to demonstrate that multiple difficult to perform experiments may be conducted on the same fresh tumor sample using an *ex-vivo* model. To demonstrate this, we selected an experiment that in humans would be prohibitively expensive to conduct but where preclinical data supports a correlation (correlation of homeoprotein Six1 expression and Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) sensitivity in ovarian cancers), and an experiment that would be ethically difficult to conduct (estrogen treatment of potentially estrogen receptor positive ovarian cancers).

METHODS: De-identified tissue samples were obtained at surgery using our IRB-approved protocol and processed using the Krumdieck tissue dissector in an *ex-vivo* system. Sections of tumor were cut to 300 μ m in thickness, transferred to 24 well plates and covered with complete RPMI1640 media or steroid deprived media depending on the study arm. After 24 hours, media was changed to control (media + vehicle) or treated with one of two treatments. Specimens were treated in triplicate with TRAIL at 50 ng/ml or 10^{-8} M 17β estradiol \pm 10^{-6} M 4-OH tamoxifen. To assay proliferation, MTS solution was added to the media for 4 hours on day 3. A 500 μ l aliquot was pipetted into each of 6 wells of a 96 well plate and read using an ELISA plate reader at 490 nm. SIX1 expression was measured by immunohistochemistry (scored as percent cells positive multiplied by intensity ranging from 1 (slight) to 3 (intense), range 0-300) and was correlated with TRAIL response by MTS assay. Estrogen receptor expression was measured by immunohistochemistry scoring as above and was compared to proliferation in response to estrogen \pm tamoxifen by measurement of percent ki67 on formalin fixed paraffin embedded sections of the *ex-vivo* slices.

RESULTS: Tumors from the omental metastases or peritoneal metastases of eleven patients were included. Histology included serous (n=6), endometrioid (n=3) and carcinosarcoma (n=2). For the TRAIL arm, 73% (8/11) were TRAIL resistant by MTS assay. Six1 homeoprotein was detected detection (score > 0) in 82% (9/11) of samples. As expected, Six1 immunohistochemistry score was significantly associated with TRAIL resistance (p= Mann Whitney U test). Specifically, the three samples that were TRAIL sensitive by MTS assay had Six1 IHC scores of 0, 0 and 15 whereas the median Six1 IHC score of the TRAIL resistant samples was 78 (range 5-210). All of the samples tested (n=8) responded to estrogen with increased Ki67, regardless of estrogen receptor status. Further analysis is ongoing.

CONCLUSION: The *ex-vivo* tissue slice model allows preclinical investigation on fresh human epithelial ovarian cancer tumor specimens as an intermediate step between *in-vitro* and murine/primate studies and when the preclinical studies may be prohibitively expensive or when clinical studies may not be ethically possible. Additional studies planned include expansion of the estrogen/tamoxifen study as well as further study of novel agents.